**University of Dundee**

**DOCTOR OF PHILOSOPHY**

# The role of pax genes in vertebrate segmentation

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*Award date:*
2010

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September 2010

# Contents

# Table of Figures

# Abbreviations

| | |
|---|---|
| AP | Antero-Posterior |
| BMP | Bone Morphogenetic Protein |
| BrdU | Bromodeoxyuridine |
| Cyp26 | Cytochrome P450 26 |
| Cdc42 | Cell Division Control Protein 42 |
| Dusp | Dual Specificity Phosphatase |
| DABCO | 1,4-diazabicyclo[2.2.2]octane |
| DAPT | N-N-3,5-Difluorophenylacetyl-L-Alanyl-S-Phenylglycine T-Butyl ester, (a.k.a. γ-Secretase Inhibitor IX) |
| DMSO | Dimethyl Sulphoxide |
| DSHB | Developmental Studies Hybridoma Bank |
| ERK | Extracellular Signal Regulated Kinase |
| ECL | Electrochemiluminescence |
| ECM | Extracellular Matrix |
| EGTA | Ethylene Glycol Tetraacetic Acid |
| EDTA | Ethylene Diamine Tetracetic Acid |
| EMT | Epithelial-to-Mesenchymal Transition |
| FBS | Foetal Bovine Serum |
| FKHR | Forkhead related protein (a.k.a. FoxO1) |
| FGF | Fibroblast Growth Factor |
| GAPDH | Glyceraldehyde 3-Phosphate Dehydrogenase |
| GFP | Green Fluorescent Protein |
| HBSS | Hank’s Balanced Saline Solution |

| | |
|---|---|
| HH | Hamburger-Hamilton stage of development (Hamburger and Hamilton, 1951) |
| Her | Hes-related protein |
| Hes | Hairy/Enhancer of Split |
| HSPG | Heparan Sulphate Proteoglycan |
| Ig | Immunoglobulin |
| IgSF | Immunoglobulin Superfamily |
| IgCAM | Immunoglobulin-containing Cell Adhesion Molecule |
| IRES | Internal Ribosome Entry Site |
| Lef | Lymphoid Enhancer-binding Factor |
| Lfng | Lunatic Fringe |
| MesP | Mesoderm Posterior (a.k.a. Meso) |
| MET | Mesenchymal-to-Epithelial Transition. |
| M-MLV RT | Moloney Murine Leukemia Virus Reverse Transcriptase |
| MOPS | 3-(N-morpholino)propanesulfonic acid |
| MRF | Myogenic Regulatory Factor |
| ML | Medio-Lateral |
| Myf | Myogenic Factor |
| MyoD | Myogenic Differentiation |
| NBT/BCIP | Nitro Blue Tetrazolium chloride/5-Bromo-4-Chloro-3-Indolyl Phosphate toluidine salt |
| NCAM | Neural Cell Adhesion Molecule |
| NECD | Notch Extracellular Domain |
| NICD | Notch Intracellular Domain |
| Pax | Paired-box |

| | |
|---|---|
| PBS | Phosphate Buffered Saline |
| PBST | PBS with 0.1% Tween20 |
| PBSX | PBS with 0.1% Triton X-100 |
| pBI | Expression plasmid with bidirectional tetracycline response element promoter |
| pCAGGS | Expression plasmid with hCMV/β-Actin Promoter |
| pCIG | pCAGGS-Ires-GFP |
| Pfu | *Pyrococcus furiosus* DNA polymerase |
| PNGase | Protein N-Glycosidase |
| pRFPRNAi | Expression plasmid containing RFP and an siRNA hairpin |
| PSA | Polysialic Acid |
| PSA-NCAM | Polysialylated Neural Cell Adhesion Molecule |
| PSM | Presomitic Mesoderm |
| Raldh | Retinaldehyde Dehydrogenase |
| RBP-Jκ | Recombinant Binding Protein Jκ (a.k.a. Su(H)) |
| Rac | Ras-related C3 botulinum toxin substrate |
| RFP | Red Fluorescent Protein (a.k.a. dsRed) |
| rtTA | Reverse tetracycline-controlled transactivator |
| ST8Sia | α2,8-sialyltransferase, (a.k.a. polysialyltransferase) |
| SHh | Sonic Hedgehog |
| Su(H) | Suppressor of Hairless (a.k.a. RBP-Jκ) |
| Taq | *Thermal aqueous* DNA polymerase |
| TBS | Tris Buffered Saline |
| TBST | TBS with 0.1% Tween20 |
| Tbx | T-Box |

# Acknowledgements

I would like to acknowledge the contribution of several researchers to this project. Firstly to my supervisor, Miguel Maroto, who guided and developed this project throughout, and to Kim Dale for additional advice and support. I would also like to recognise the contribution of Ella Rosenzweig, who pioneered this project in the Maroto lab. The contributions of Ram Reshef and Angela Nieto were also greatly appreciated.

Additionally, a number of labs graciously donated materials for this project: Yoshiko Takahashi and Nick Van Hateran for DNA constructs and Sylvie Dufour and the Developmental Studies Hybridoma Bank for antibodies.

This work would also not have been possible without the help, advice and support of members of the various laboratories in the Division of Cell and Developmental Biology at Dundee, specifically the labs of Kim Dale, Kees Weijer, Kate Storey, Cheryll Tickle and Inke Nathke. I'd also like to recognise the support of Calum Thompson and Sam Swift at the Light Microscopy Facility.

Finally, I'd like to acknowledge the enormously valuable support, advice and training from all the members of the Maroto and Dale labs during this project, especially Dr. Shona Gray, Dr. Sarah Gibb, and Dr. Zoltan Ferjentsik.

David Wright

# Declarations

## Candidate Declarations

I declare that I am the author of this thesis, and unless otherwise stated I have consulted all references cited. Unless otherwise stated, the work presented here is a record of my individual work, and this work has not previously been accepted for a higher degree.

Signed,

David Wright

## Supervisor Statement

The conditions of the relevant Ordinance and Regulations have been fulfilled.

Signed,

Miguel Maroto

David Wright

# Abstract

Pax genes are involved in a range of processes in the developing embryo. *Pax3* and *Pax7* in particular are associated with the paraxial mesoderm, especially the development of the myogenic lineage. However, recent studies have suggested that *Pax3* and *Pax7* may have earlier, morphogenetic roles in the segementation of the paraxial mesoderm. Here we examine the expression of *Pax3* and *Pax7* in the chicken presomitic mesoderm, and investigate their function using an in ovo electroporation transfection technique. We find that *Pax3/7* drives precocious differentiation of PSM towards the myogenic lineage, as well as inducing a range of morphological changes in PSM tissue. These changes include alterations in the cytoskeleton, cell adhesion, and extracellular matrix formation.

# Introduction

## Segmentation

Segmentation is the formation of repeated segmental units in the body plan of animals. While several distinct systems of segmentation have been described in invertebrates, vertebrates form their segmental units by somitogenesis, whereby the embryo produces transient epithelial segments which either give rise to or pattern all of the segmental structures of the body.

### Paraxial Mesoderm

Vertebrate embryos produce the embryonic mesoderm through the process of gastrulation (fig. 1.1a). Cells in the epiblast undergo an epithelial-to-mesenchymal transition at the primitive streak, and then undergo differential migration to form the different mesodermal lineages. The antero-posterior position of ingressing cells along the streak appears to correlate with the medio-lateral distribution of their derivative tissues. Thus, the axial mesoderm of the notochord is produced by the node (called Hensen's node in the chicken) which lies at the anterior of the primitive streak, the paraxial mesoderm which lies adjacent to the neural tube is produced by the rostral region of the streak, and the intermediate and lateral plate mesoderm which are positioned more laterally are produced by the caudal region of the primitive streak (fig. 1.1c; Psychoyos and Stern, 1996). However, the primitive streak is a transient structure within the embryo, and disappears before axis elongation is complete; cells in the caudal embryo give rise to

tailbud, an undifferentiated tissue which produces the mesoderm and neural tube in the caudal embryo (fig. 1.1d; Catala *et al.*, 1995). This system of progressive axis elongation sets up a gradient of differentiation, where cells at the rostral end of the embryo are undergoing maturation and differentiation while stem cell populations at the caudal end are giving rise to undifferentiated tissue at the tail end. This is most clearly evident in the process of segmentation, where new segments appear sequentially from the primordial unsegmented tissue with a regular, species specific rhythm.

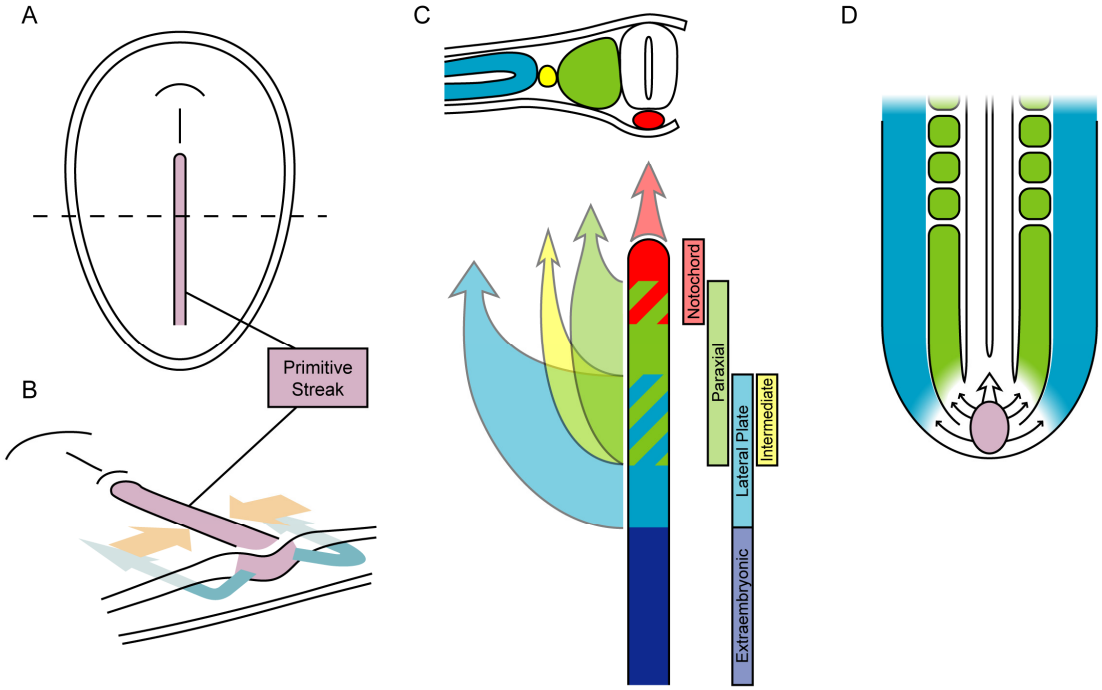


**Figure 1.1: Formation of the Paraxial Mesoderm**

A-B: Mesoderm is formed at gastrulation. In the chick, cells in the epiblast move towards the primitive streak (B, orange arrows, primitive streak shown in pink), and delaminate to form the mesoderm (B, blue arrows). C: During primary body extension, the mesoderm is derived from the primitive streak. Cells mediolateral identity is correlated with their anteroposterior position in the primitive streak. D: During secondary body extension, the mesoderm is derived from the tailbud (pink). (B, after Gilbert, 2006; C, after Gray and Lewis, 1918 and Psychoyos and Stern, 1996; D, after Catala *et al.*, 1995).

The primary segmental tissue is the paraxial mesoderm, two bilateral rods of tissue which lie adjacent to the neural tube. At the caudal end, this tissue does not exhibit overt segmentation, and is referred to as the presomitic mesoderm (PSM). Initially, this tissue is a loose mesenchyme, but as development progresses it condenses and begins to acquire epithelial character (Christ and Ordahl, 1995). A network of signalling events and transcriptional feedback loops in the caudal PSM prefigures a segmental pattern (Dubrulle *et al.*, 2001). As the PSM matures, these presumptive

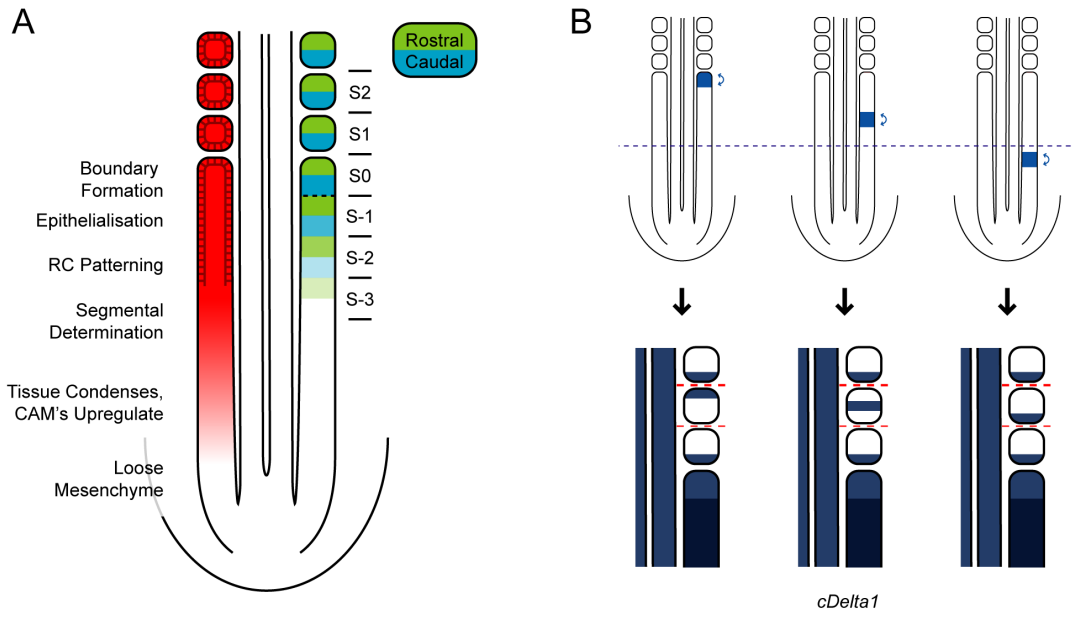


**Figure 1.2: Maturation and Segmentation in the Presomitic Mesoderm**

The Paraxial Mesoderm has a rostrocaudal gradient of differentiation (A, left half). During development, sharp boundaries are established between "rostral" (green) and "caudal" (teal) identity genes, which give rise to segment boundaries (A, right half, after Dubrulle and Pourquie, 2004). B: At a point in the mid-PSM, segmental polarity is determined, and surgically inverted somitomeres maintain their RC identity. Below this level, they attain the correct identity from the surrounding tissue (B, after Dubrulle *et al.*, 2001).

segments acquire antero-posterior (AP) polarity and form sharp gene expression boudaries between neighbouring segments. These presumptive segments are called somitomeres. Ultimately, cell signalling across this boundary causes cell motility events that physically separate this segment from the PSM, forming a physical somite. The somite rapidly obtains a polarised epithelial structure, leaving only a few mesenchymal cells at the centre, in a structure called the somitocoel (fig. 1.2a; Christ and Ordahl, 1995).

Epithelial somites are transient structures within the embryo, and cells progressively undergo an epithelial-to-mesenchymal transition as they differentiate (fig. 1.3). Signals from the surrounding tissues, particularily the notochord and floorplate, dorsal neural tube, surface ectoderm and lateral plate mesoderm, combine with the somites intrinsic AP identity to divide the somitic mesoderm up into morphogenetic domains. This is most dramatic in the ventral somite, which loses its epithelial character to form the sclerotome. The sclerotome is then patterned by intrinsic AP identity and signals from surrounding tissues to produce the axial skeleton; the ribs and vertebrae (Christ *et al.*, 2004). The segmental pattern of the sclerotome is also important for segmental patterning the peripheral nervous system as the anterior domain forms a substrate for neurite outgrowth from the neural tube, as well as the migratory pathways for neural crest cells and neurons producing the dorsal root ganglia (Keynes and Stern, 1988). Interestingly, the sclerotome undergoes a second segmentation event called resegmentation,

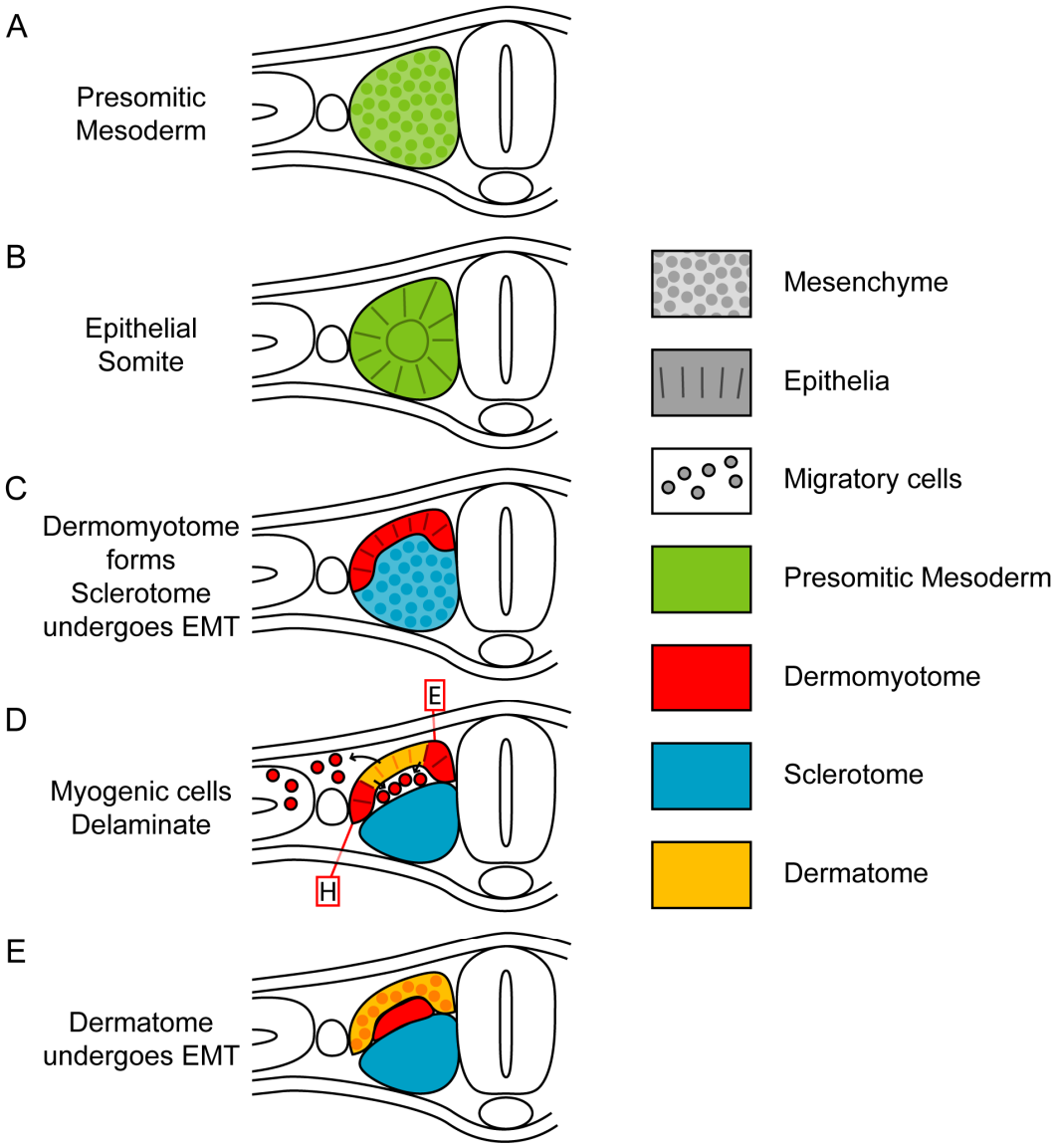


**Figure 1.3: Development of the Paraxial Mesoderm**

The caudal, unsegmented paraxial mesoderm (PSM, green) has a mesenchymal character (A). When segmentation occurs, the paraxial mesoderm exhibits a transient epithelial structure, the somite (B). The somite is divided into the dorsal dermomyotome (red) and the ventral sclerotome (blue, C). Waves of myogenic cells delaminate from the dermomyotome, and form the myotome within the paraxial mesoderm and the skeletal muscles of the body (D). Myogenic domains persist in the extremities of the epithelial somite, known as the Epaxial *(E)* and Hypaxial *(H)* dermomyotome. Ultimately, the dermatome (orange) loses its epithelial character (E).

however the resegmentation boundary occurs within the somite. The upshot of this is that each vertebral unit is composed of parts of two adjacent somites (Christ *et al.*, 2004).

The remaining epithelial structure is called the dermamyotome, which is similarily compartmentalised into epaxial (medial), central and hypaxial (lateral) domains (fig. 1.3c,d). Cells within the dermamyotome undergo waves of delamination to form the precursors of the skeletal muscles. Cells migrate ventrally to form the myotome, with cells in leaving the epaxial domains to form the deep muscles of the back, and the hypaxial domain to form superficial muscles of the ventrum and body wall (Denetclaw *et al.*, 1997; Denetclaw and Ordahl, 2000). Also, signals from the limb buds cause the delamination and long range migration of a subset of hypaxial dermamyotome cells which become the muscles of the limbs (Williams and Ordahl, 1994). A population of hypaxial dermamyotome cells also migrate away from the somites to form the muscles of the diaphragm and tongue (Bladt *et al.*, 1995; Zhou *et al.*, 2008; Li *et al.*, 1999).

After the last myogenic cells have left, all that remains is the dermatome, which looses it's epithelial structure and produces the dermis of the back (fig. 1.3e; Brill *et al.*, 1995).

## The Segmentation Clock

Early attempts to define the mechanisms of vertebrate segmentation supposed that the PSM had a predefined segmental organisation which only became apparent as cells produced intersomitic borders, but could not explain how this segmental organisation arose in the first place (Dale and Pourquie, 2000). Cooke and Zeeman suggested that oscillations in the caudal PSM combined with a regressing wavefront of differentiation as the axis extends provided a theoretical model for producing this segmental organisation (fig. 1.4; Cooke and Zeeman, 1976). In 1997, the first evidence for such an oscillator was discovered in the form of *Hairy1* (Palmeirim *et al.*, 1997), a transcription factor related to the Drosophila pair-rule gene *Hairy*, which exhibited a cyclic expression pattern with the same period as somitogenesis (90 minutes in chick). Other Hairy/Enhancer of Split family genes such as *Hes1* (Jouve *et al.*, 2000) and *Hes7* (Bessho *et al.*, 2001) in mouse and *Her1* (Holley *et al.*, 2000) in zebrafish were identified. This gene family is a downstream target of Notch signalling, and the identification of the Notch pathway modulator *Lunatic Fringe* (*Lfng*) as another oscillating gene suggested that Notch signalling may be a core component of the segmentation clock (McGrew *et al.*, 1998). Components of other signalling pathways, including Wnt components such as *Axin2* (Aulehla *et al.*, 2003) and FGF components such as *Dusp4* (Niwa *et al.*, 2007) have also been identified as cycling in some species. However, it should be borne in mind that there are some species differences in oscillating gene expression, for example

*DeltaC* oscillates in zebrafish but not other species (Jiang *et al.*, 2000) and *Axin2* does not cycle in the chicken (Gibb *et al.*, 2009).

The precise nature of the wavefront which is suggested to transform cyclic activity in the caudal PSM into a segmental pattern has not been clearly

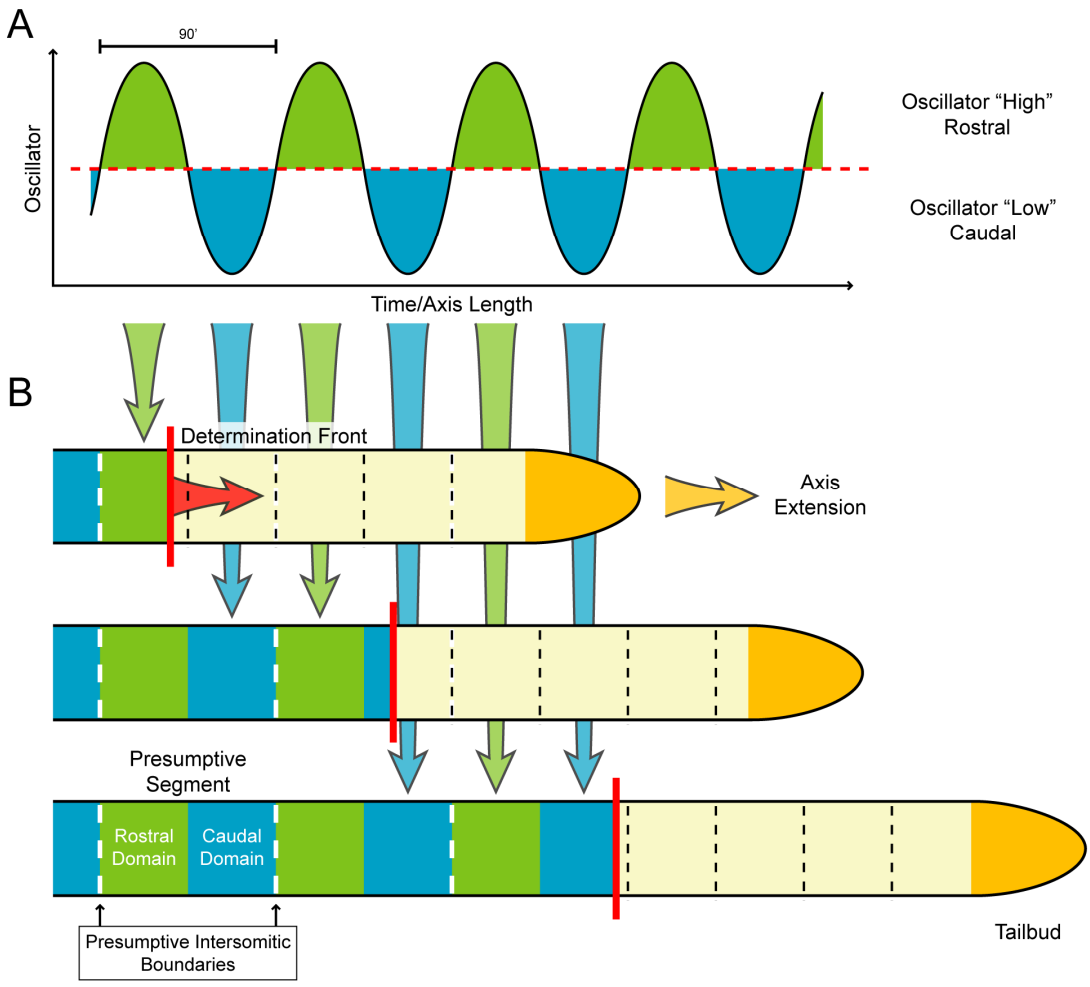


**Figure 1.4: The Segmentation Clock**

A: According to the "clock-and-wavefront" model, segmentation is driven by an oscillator acting in the PSM with the same period as somite production. B: As the axis extends and new tissue is laid down, a "wavefront" of differentiation termed the determination front (red bar) moves along the tissue. Depending on the polarity of the oscillator, tissue is assigned to a "rostral" (green) or "caudal" (teal) fate. Each pair of rostrocaudal units represents a presumptive segment (B, after Dale and Pourquie, 2000).

defined, although it is thought to involve an antagonistic gradient between Retinoic Acid (RA) and FGF signalling (Diez del Corral *et al.*, 2003). FGF signalling in particular is thought to control recruitment of cells into each somitomere, giving rise to the concept of the "determination front" (Dubrulle *et al.*, 2001). However, because of the important role of FGF in PSM maturation (Delfini *et al.*, 2005), the FGF gradient should not be solely considered in terms of anterior-posterior identity determination.

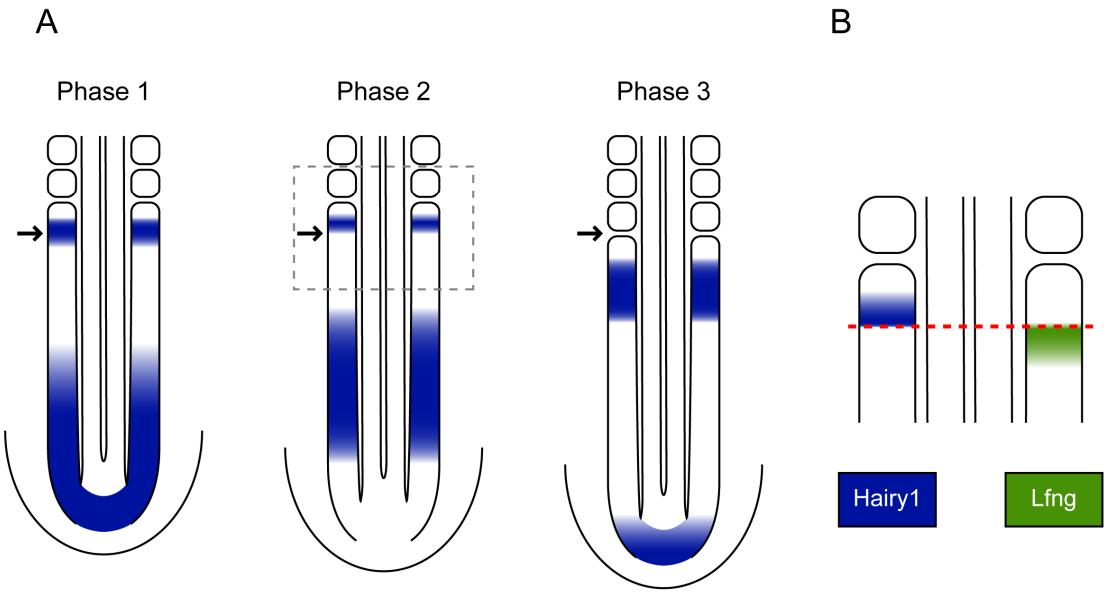


**Figure 1.5: Oscillation and the Intersomitic Boundary**

Activated Notch signalling, as assayed by expression of *Hairy1* in chick and NICD in mouse, goes through characteristic waves of expression in the PSM with the same periodicity as somite formation. Each oscillation can be broken down into three phases (A, phases of *Hairy1* expression, after Palmeirim *et al.*, 1997). In the rostral PSM, the posterior boundary of active Notch signalling corresponds to the presumptive intersomitic boundary (B, after Maroto *et al.*, 2005). Interestingly, *Lfng*, a negative modulator of Notch which is positively regulated by Notch signalling, oscillates in the PSM, and closely resembles *Hairy1* in most respects, is localised caudal to the presumptive intersomitc boundary.

## The Rostral PSM

Whatever its role in determining somite size, the PSM undergoes a dramatic change rostral to the determination front. The period of Notch signalling oscillation sharply extends, putting it out of phase with the caudal oscillations and giving rise to the characteristic wavelike pattern (fig. 1.5, Maroto *et al.*, 2005). The ratio of active to inactive Notch signalling in each cycle also increases, until it is activated at the nascent somite border for nearly a whole cycle before being permanently inactivated. The transcription factor profile also changes, with transcription factors such as *Paraxis* (Barnes *et al.*, 1997) *Pax3* (Schubert *et al.*, 2001) and *Tbx18* (Tanaka and Tickle, 2004) becoming upregulated, as well as signalling components such as *EphA4* and *EphrinB2* (Baker and Antin, 2003). AP polarity is established (fig. 1.2b, Dubrulle *et al.*, 2001). Cell adhesion molecules such as NCAM and N-cadherin are upregulated, and the mesenchyme condenses and begins to assume an epithelioid structure at the periphery (Duband *et al.*, 1987), and begin the formation of the somitic epithelium at the medial surface (Martins *et al.*, 2009). *Integrin α5* transcription is downregulated in this domain, suggesting an alteration of cell-matrix interactive behaviour (Rifes *et al.*, 2007).

## Epithelialisation

An important aspect of segmentation is the formation of a polarised epithelium. Rather than an instantaneous transition at the point of boundary formation, the process of epithelialisation begins several hours before the

somite buds off from the PSM, and is not fully completed until some time after (Dubrulle *et al.*, 2001; Martins *et al.*, 2009). Early observations suggest that the PSM initially forms a loose mesenchyme, which undergoes a condensation (Duband *et al.*, 1987) when it reaches the determination front (Dubrulle *et al.*, 2001). Cells in the centre of the PSM maintain a mesenchymal identity, while those at the periphery start to assume an epitheloid organisation (Duband *et al.*, 1987). Recent observations using confocal microscopy of the intact PSM showed that cells at the medial boundary of the PSM also alter their morphology to form a simple cuboidal epithelium from around S-2 (Martins *et al.*, 2009). When boundary formation

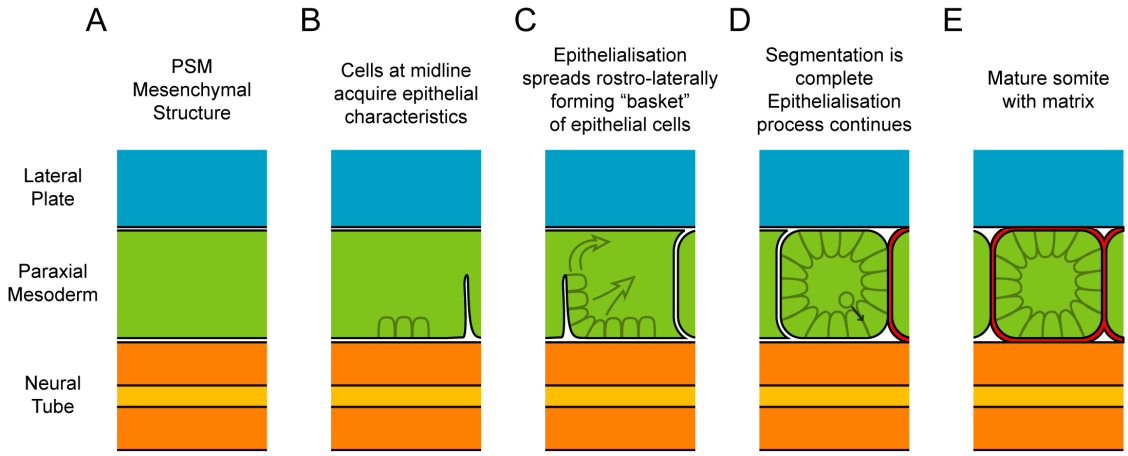


**Figure 1.6: Assembly of Somitic Epithelium**

Somitic epithelialisation is a gradual process beginning in the anterior PSM and continuing into the early somite. The first epithelioid cells are detected in the medial PSM (i.e. adjacent to the neural tube, shown in orange) around S-2 (B). A definitive epithelium is assembled by S0, and cells are progressively added into the epithelium from the medio-caudal end of the presumptive somite to the rostro-lateral (C, arrows). Upregulation of CAMs during this process gives rise to an "adhesion basket". As the intersomitic gap is formed, only the caudal part of the somite (shown on the left in C) is epithelial. Even after a the epithelial somite has formed (D), mesenchymal cells are still added to the epithelium from a pool of cells in the somitic core. Eventually, a mature somite forms with a complete extracellular matrix (E). (After Martins *et al.*, 2009).

occurs, cells become organised into the epithelium on the rostral side of the boundary (Kulesa and Fraser, 2002; Nakaya *et al.*, 2004). Cells appear to be sequentially recruited into the epithelium and establish a spindle-like structure with actin and N-cadherin accumulating at the apical surface, and assemble in a caudo-medial to rostro-lateral direction. Cells are also recruited from the somitocoel mesenchyme, so that as the somite matures it becomes increasingly epithelial (Martins *et al.*, 2009).

The somitic epithelium is pseudostratified, and cell bodies move between epithelial layers as they progress through the cell cycle (Bellairs, 1979; Langman and Nelson, 1968). It's maturation and polarity has been shown to depend on fibronectin (Rifes *et al.*, 2007; Martins *et al.*, 2009).

The formation of polarised epithelia involves dramatic cytoskeletal rearrangements, changes in cell adhesion and cell matrix interactions, and the development of a subcellular organisation epitomised by the formation of apico-basal polarity. Interestingly, the Rho family of small GTPases is implicated in all of these events, and consequently are important candidates for regulators of epithelialisation (Van Aelst and Symons, 2002). These molecules form an important second messenger system for a range of inputs, including cytokines, growth factors and even cell adhesion molecules, and relay these signals to a range of biological processes, including the cytoskeleton (Van Aelst and D'Souza-Schorey, 1997; Hall, 1998). Two members of the Rho family, *Rac1* and *Cdc42*, have been specifically

implicated in somite epithelialisation (Nakaya *et al.*, 2004). In contrast, the small GTPase *RhoA* is not only absent in the PSM, cells transfected with *RhoA* rapidly undergo apoptosis (Watanabe *et al.*, 2007). *Rac1* and *Cdc42* are thought to have critical functions in embryonic morphogenesis, and mice lacking either of them die very early from cytoskeletal defects (Chen *et al.*, 2000; Sugihara *et al.*, 1998). A switch from high to low levels of *Cdc42* activity was required for the initiation of MET within the embryo, while in general the opposite was true in *Rac1*; however, when *Rac1* activity was perturbed cells were aberrantly formed, suggesting it may have a role in cell polarity and the localisation of cytoskeletal and cell adhesion components (Nakaya *et al.*, 2004). Interestingly, *Cdc42* appears to be controlled by Eph-Ephrin reverse signalling (Watanabe *et al.*, 2009), while *Rac1* appears to mediate the role of *Paraxis* in driving epithelialisation (Nakaya *et al.*, 2004).

# Signalling Pathways

Multiple signalling pathways are implicated in the development of the paraxial mesoderm and the process of segmentation. Some of these signalling pathways, chiefly the Notch pathway, are integral to the “segmentation clock” which establishes the segmental pattern in the PSM. While components of the FGF and Wnt pathways have been implicated in the segmentation clock, they also have roles in the maturation of the paraxial mesoderm along the AP axis. Additionally, signalling via ephs and ephrins is thought to be important in establishing and maintaining segmental borders in the rostral PSM.

## Notch Signalling

Notch signalling is an important cell signalling pathway in development (fig. 1.7). Notch receptors are single-pass transmembrane proteins. Their ligands are also membrane-bound, and belong to the Delta and Serrate families. Upon ligand binding, a series of proteolytic cleavage events occur, culminating in the intramembrane cleavage of Notch by the γ-secretase complex (fig. 1.7c). The intracellular fragment of Notch (NICD) then translocates to the nucleus, where it forms a complex with *Suppressor of Hairless* (*Su(H)*, also known as *RBP-Jκ*) to alter transcription (fig 1.7d). Notch is subject to modulation based on it's glycosylation state, which is regulated by the expression of Fringe glycosyltransferases, such as *Lunatic Fringe* (*Lfng*; Fortini, 2009; fig 1.7b). In many contexts, Fringe glycosylation has been shown to improve the efficacy of Notch-delta signalling (Panin *et al.*, 1997), however in the PSM it has clearly been shown to act as a repressor of Notch-delta signalling (Dale *et al.*, 2003).

The Notch pathway has long been associated with somitogenesis, as mutants for pathway components show defects in segmentation. The first was *Pudgy* (Grüneberg, 1961), an allele of *Delta3* which exhibit profoundly disrupted vertebral segmentation without affecting differentiation of the paraxial mesoderm. All other Notch pathway mutants are embryonic lethal, with varying degrees of somitic defects. *Delta1* mutants produce a few irregular somites in the rostral end of the embryo, and no anterior-posterior polarity could be identified (Hrabe de Angelis *et al.*, 1997). *Notch1* knockout mice

produce a few disrupted segments (Swiatek *et al.*, 1994), as do mutants of the downstream effector *RBP-Jκ* (Oka *et al.*, 1995), however due to potential redundancy issues none of these should be considered a true Notch signalling knockout. Double knockouts of the two presenilin enzymes which make up the catalytic core of the γ-secretase complex required for ligand-induced cleavage of the Notch receptor represent the most complete Notch null phenotype; these mice do not produce any segments at all (Donoviel *et al.*, 1999, Ferjentsik *et al.*, 2009).

Since most of the core Notch components are expressed at a constant level throughout the PSM and are not known to undergo dynamic regulation, the Notch pathway was not an immediate candidate for the temporal regulation of segmentation. However, downstream targets of Notch such as *Hairy1* (Palmeirim *et al.*, 1997) and *Lfng* (McGrew *et al.*, 1998), and ultimately Notch signalling itself (as assayed by cleaved NICD, Morimoto *et al.*, 2005) were observed to have a profoundly dynamic expression pattern in the PSM, which repeated itself precisely in phase with the production of new somites (fig. 1.5a). This behaviour is an intrinsic property of the PSM, as explants removed from the embryo continue to undergo cycles of Notch activation, and even when small fragments of the PSM are isolated they still undergo oscillations in phase with the intact tissue (Maroto *et al.*, 2005). The role of *Lfng* in this system was demonstrated in the chick, where *Lfng* negatively

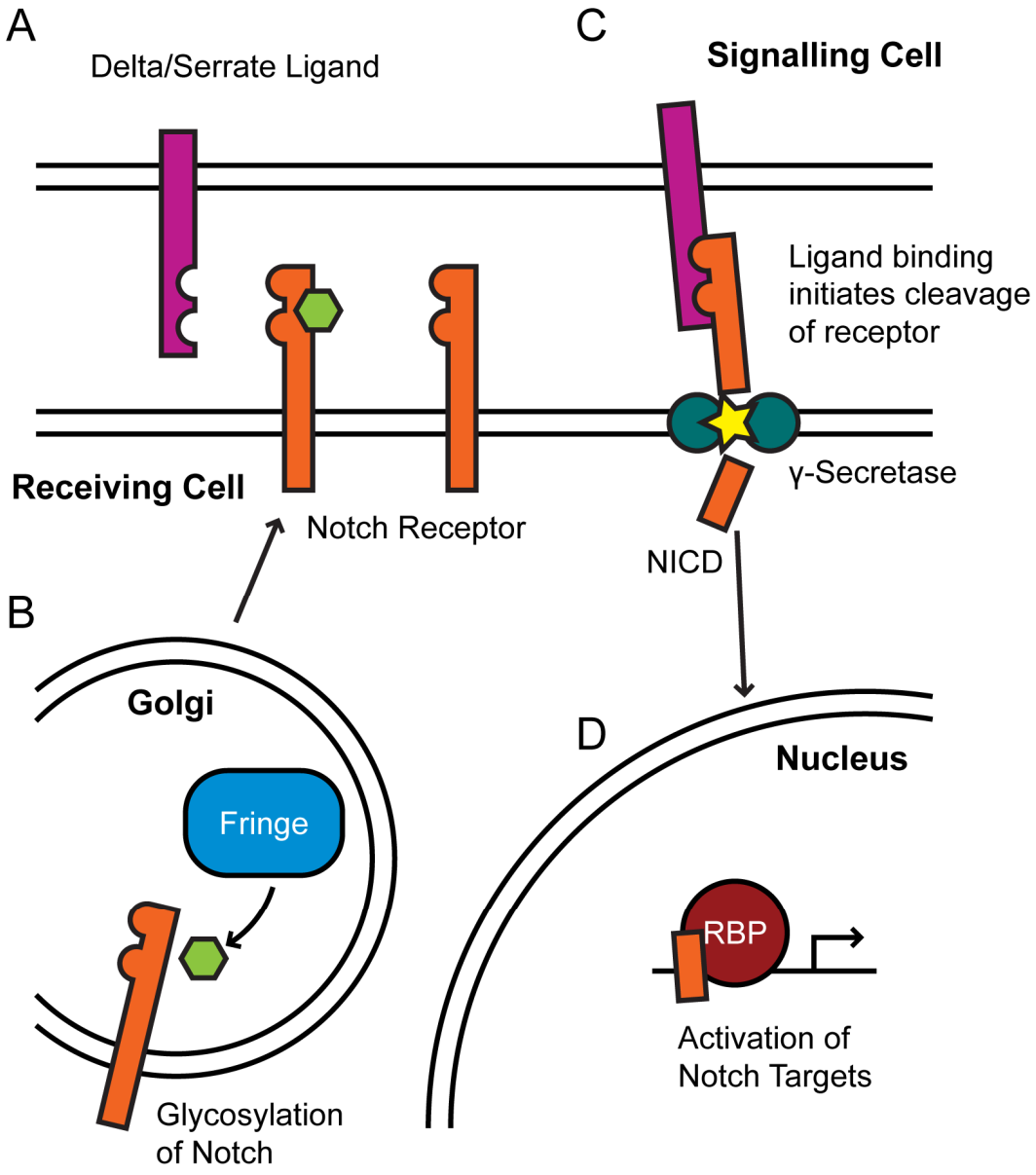


**Figure 1.7: Notch Signalling Pathway**

Notch is a contact-binding signalling pathway. Signalling cells produce membrane-bound ligands of the Delta and Serrate families (A, purple). Receiving cells express the Notch receptor (orange) which may be post-translationally modified by glycosyltransferases such as Fringe (B, blue). This glycosylation (green hexagon) modulates ligand-receptor interactions, and in the PSM functions to interrupt Notch-Delta signalling (Dale *et al.*, 2003). Upon activation (C), γ-secretase (teal) cleaves the Notch receptor to release an intracellular fragment (NICD), which translocates to the nucleus (D) and affects gene expression by modifying the function of RBP-jκ.

regulates Notch signalling, presumably by modulating ligand-receptor interactions (Dale *et al.*, 2003). Mice lacking *Lfng* exhibit disrupted segmentation and polarity, although segment boundaries do form at irregular intervals (Zhang and Gridley, 1998; Evrard *et al.*, 1998). Recent work has shown that Notch signalling undergoes oscillations even in the absence of *Lfng*, although the expression domains are less well defined (Ferjentsik *et al.*, 2009).

However, while the a correlation between *Lfng* and other targets of Notch signalling such as Hairy genes is maintained throughout most of the PSM, it diverges at the level of the forming segmental border (Maroto *et al.*, 2005). This was confirmed in mouse, where it was shown that *Lfng* is upregulated in the rostral compartment of S-1, and activated Notch signalling is detected in the adjacent caudal compartment of S0, with a sharp boundary between the two (Morimoto *et al.*, 2005). The functional importance of this boundary was demonstrated in the chick by grafting cells transfected with NICD into the rostral PSM of an untransfected host, resulting in an ectopic segment boundary between the transfected and untransfected tissue (Sato *et al.*, 2002). Interestingly, the same effect was observed when using *Lfng* instead of NICD, which the authors erroneously assumed was a positive regulator of Notch signalling. In reality, *Lfng* negatively regulates NICD (Dale *et al.*, 2003), so this effect is presumably a result of setting up a sharp boundary between transfected and untransfected tissue.

A requirement for Notch signalling in order to form segment borders appears to be supported by pharmacological disruption of Notch signalling; when mouse embryos are treated with small molecule γ-secretase inhibitors such as DAPT or LY411575, they usually form one or at most two segments before boundary formation halts (Ferjentsik *et al.*, 2009). A subtly different effect was observed in chick embryos, where two to six irregular, not bilaterally paired segment boundaries formed in the absence of Notch (Gibb, 2009). The mechanism by which a sharp Notch signalling boundary drives segmentation has not been directly explored, but it is known that *EphrinB2* is a direct target of Notch1 signalling in the endocardium of the developing heart (Grego-Bessa *et al.*, 2007), that *EphrinB2* is upregulated in the NICD-positive posterior

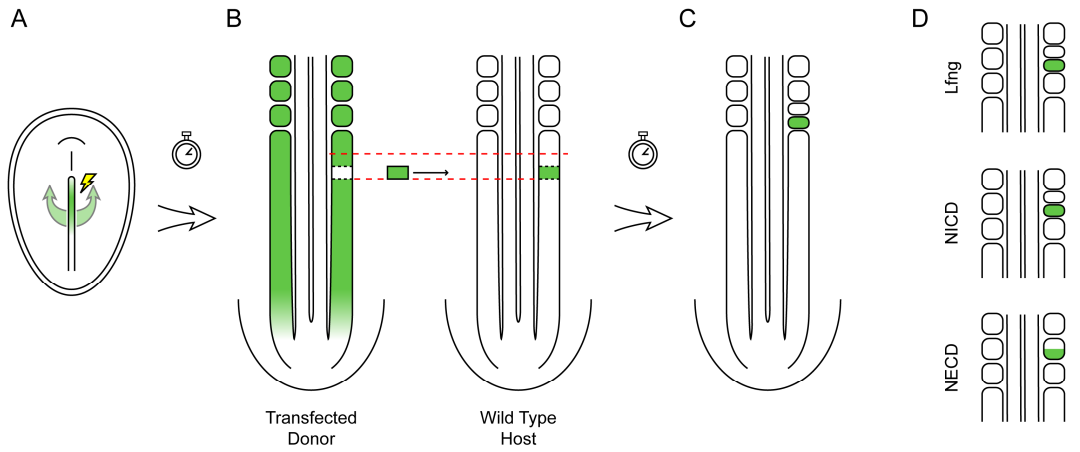


**Figure 1.8: Gap Formation Assay**

Gap formation assay (Sato *et al.*, 2002) developed to identify factors involved in establishing the intersomitic gap. Embryos are transfected and grown to an appropriate stage (A). Transfected donor tissue from S-1 is homotopically transplanted into a host embryo, producing a sharp border of transfected-untransfected tissue in the middle of the presumptive somite (B). Host is grown until tissue is incorporated into somites (C). Constructs which are involved in segmentation, such as *Lfng* and activated Notch (NICD) induce an extopic somitic gap. The Notch extracellular domain (NECD), which is incapable of initiating cell signalling, does not produce an ectopic gap (D).

somite during boundary formation (Baker and Antin, 2003), that and *EphrinB2* signalling is sufficient to drive segmentation (Watanabe *et al.*, 2009).

## Eph-Ephrin Signalling

Eph receptors are a family of transmembrane receptor tyrosine kinases which are involved in a range of border formation and cell sorting functions (Xu *et al.*, 2000). Originally classed as orphan receptors, their ligands were eventually identified and termed ephrins. The ligands fall into two classes; GPI-linked "A" type receptors and transmembrane "B" type receptors (Gale *et al.*, 1996). Eph receptors can also be generally grouped by their affinity for each ligand class, although some receptors can bind across classes. Interestingly, in the case of EphrinB ligands, the distinction between receptor and ligand is blurred as these proteins can function as receptors in much the same way as the ephs (Bruckner *et al.*, 1997). Consequently, signalling through the eph receptor is referred to as "forward" signalling, while signalling through the EphrinB ligands is referred to as "reverse" signalling (fig. 1.9a).

Eph signalling is generally associated with contact repulsion. In exploratory cell behaviours such as axonal guidance or cell migration, activation of eph receptors results in a localised collapse of the actin cytoskeleton, biasing migration away from the ligand (Harbott and Nobes, 2005; fig. 1.9b). On a tissue scale, this function can prevent intermingling between adjacent domains (Mellitzer *et al.*, 1999; fig. 1.9c), and expression patterns of ephs and their complimentary ephrins often form strikingly exclusive domains in

development (Gale *et al.*, 1996). However, it has also been suggested that eph-ephrin signalling may regulate adhesion (Holmberg and Frisen, 2002). This combination of contact repulsion and differential adhesion is thought to underlie boundary formation and maintenance in the nervous system (Cooke *et al.*, 2005).

*EphA4* (Nieto *et al.*, 1992, Baker and Antin, 2003) and *EphrinB2* (Bergemann *et al.*, 1995, Baker and Antin, 2003) are transiently expressed in tightly apposed domains in the rostral PSM during boundary formation. *EphrinB2* is expressed in the posterior compartment of the nascent somite, and *EphA4* in the anterior compartment of the subsequent segment. Expression of both factors persists for longer than the the clock period, resulting in a banded pattern in the newly formed somites. In some cases, a diffuse staining of both factors can be detected in S-1 and S-2. In the chick, *EphA4* is a downstream target of *MesP* transcription factors (Nakajima *et al.*, 2006; Watanabe *et al.*, 2009), while *EphrinB2* may be a direct target of Notch signalling (Grego-Bessa *et al.*, 2007).

Functional evidence that eph-ephrin interactions are important in segmentation first came from studies in zebrafish. Disruption of eph-ephrin expression blocked the formation of an intersomitic boundary and consequent epithelialisation (Durbin *et al.*, 1998). Transplantation of *EphA4* expressing cells into a mutant which fails to establish a sharp *EphA4-EphrinB2*

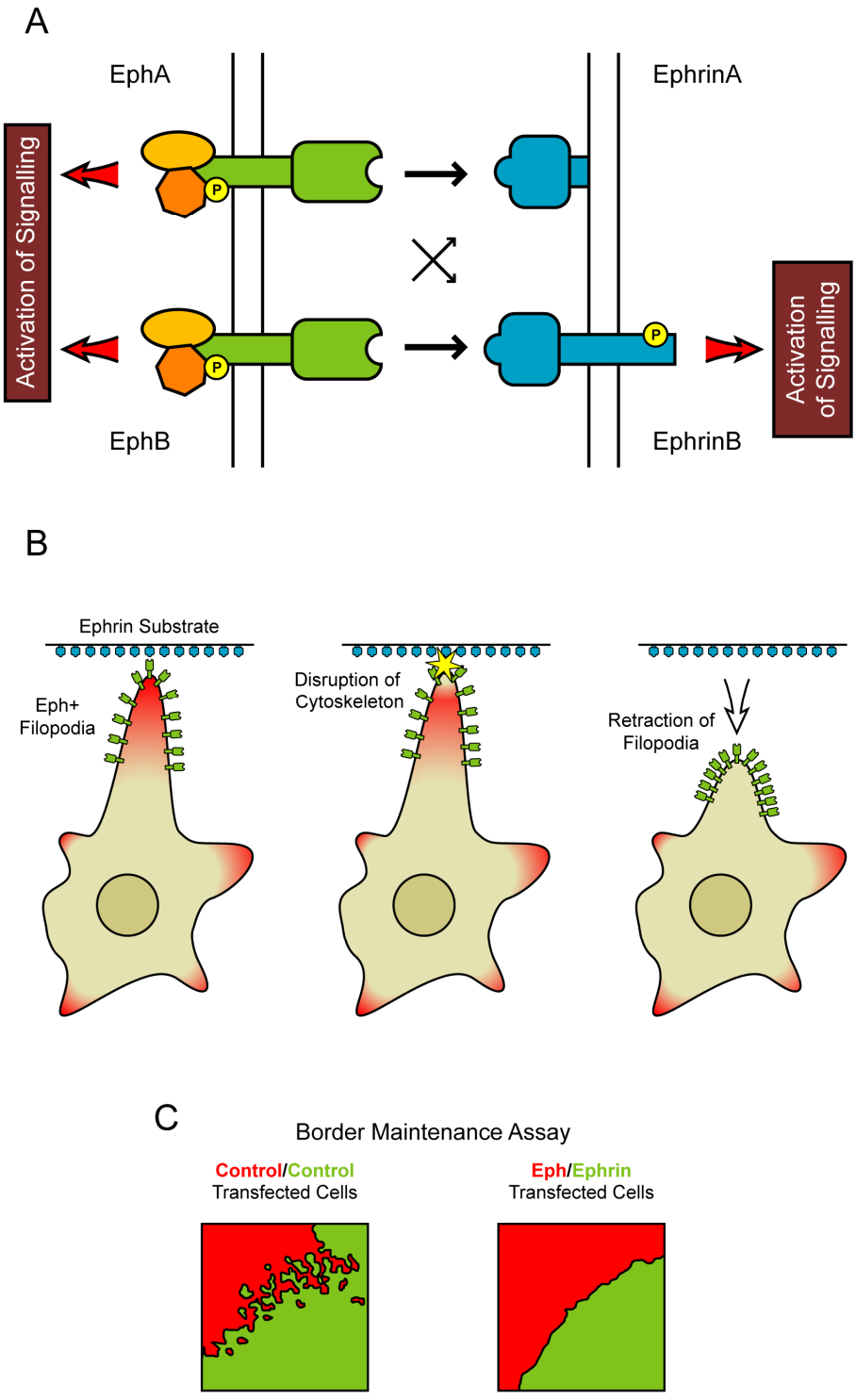


**Figure 1.9: Eph-Ephrin Signalling**

A: Two families of ephs and ephrins have been described, based on the structure of the ephrin ligand. EphrinA ligands are membrane-linked proteins, while EphrinB

**cont. overleaf**

boundaries (and lacks segmental borders) was sufficient to drive not only segmentation, but epithelialisation in transfected tissue (Barrios *et al.*, 2003). These observations were extended in the chick using the transfection-graft assay to introduce sharp borders of either *EphA4* for *EphrinB2*, resulting in formation of an ectopic somite boundary (Watanabe *et al.*, 2009). By analysing mutant forms of the ligand and receptor, it was shown that this function is due to reverse signalling via *EphrinB2*.

However, while *EphrinB2* knockout mice exhibit abberant segment polarity, a segmental pattern is still produced. Furthermore, a mutant form of *EphrinB2* which lacks autophosphorylation sites was capable of rescuing the segmental phenotype completely, suggesting that the defect was not due to reverse signalling (Davy and Soriano, 2007). This could potentially be explained by functional redundancy, as *EphrinB1* is expressed in a similar domain.

The ability of *EphrinB2* to produce a boundary appears to be dependent on

**Figure 1.9: Eph-Ephrin Signalling cont.**

ligands are transmembrane proteins which may be involved in reverse signalling. While receptors tend to be activated by their own family of ligand, cross-family binding has been described, e.g. in the case of EphA4-EphrinB2 (after Holder and Klein, 1999). B: Eph-ephrin signalling is thought to regulate exploratory cell behaviours. Contact between eph-positive filopodia and repulsive, ephrin-positive substrate causes a localised collapse of the cytoskeleton and retraction of the cellular process. C: This behaviour, combined with regulation of differential adhesion, can lead to the inhibition of cell mixing in *in vitro* assays (Mellitzer *et al.*, 1999). Recombinant cultures of cells transfected with compatible ephs and ephrins do not intermix, but maintain sharp boundaries between the two populations.

inactivation of the Rho family GTPase *Cdc42* (Watanabe *et al.*, 2009), known to mediate the switch between mesenchymal or epithelial cell identity (Nakaya *et al.*, 2004). While this pathway appears to be required for border formation in the grafting assay, other functions of eph-ephrin signalling may be involved in segmentation and epithelialisation. *Rac1*, another Rho family GTPase important in somite epithelialisation (Nakaya *et al.*, 2004) is controlled by eph-ephrin signalling (Shamah *et al.*, 2001). It has been suggested that eph-ephrin signalling may modulate the activity of cell adhesion molecules such as NCAM and N-cadherin (Kasemeier-Kulesa *et al.*, 2006; Cooke *et al.*, 2005); and computer modelling has suggested that this may be sufficient to drive intersomitic gap formation (Glazier *et al.*, 2008). Also, eph-ephrin signalling has been shown to positively and negatively regulate integrin activity in different contexts (Zou *et al.*, 1999; Miao *et al.*, 2000; Gu and Park, 2001). It has recently been shown that the function of integrins and their extracellular matrix ligands fibronectin are also important in segmentation (Rifes *et al.*, 2007, Martins *et al.*, 2009).

## Other Signalling Pathways

Other signalling pathways are involved in the patterning of the paraxial mesoderm. The tailbud is a source of Wnt3a and FGF8, which are thought to be important in maintaining the progenitor pool during axis elongation (Greco *et al.*, 1996; Diez del Corral *et al.*, 2003; fig. 1.10a). Readouts of Wnt and FGF signalling, stabilised β-catenin and phosphorylated ERK respectively, are expressed in a gradient along the PSM (Aulehla *et al.*, 2008; Delfini *et al.*,

2005). Activity of FGF8 is thought to be antagonised by the vitamin A derivative retinoic acid (RA; Diez del Corral *et al.*, 2003; fig. 1.10a). RA synthesis enzymes (retinaldehyde dehydrogenases, Raldh) are expressed in the mature somites, while the tailbud expresses RA degradation enzyme Cyp26. This establishes an opposing gradient of RA activity. These factors have classically been assumed to regulate the maturation of the PSM, although it has recently been observed that components of the Wnt and FGF pathway have oscillatory expression in the caudal PSM (Dequeant *et al.*, 2006). Additionally, the Wnt pathway has been implicated in the regulation of

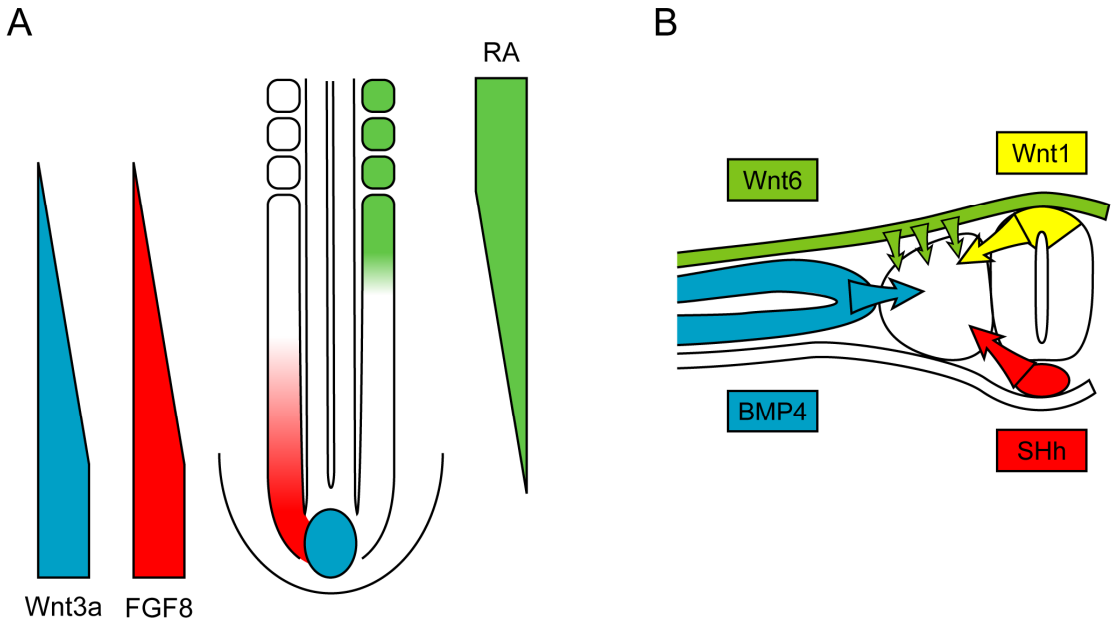


**Figure 1.10: Signalling in the Paraxial Mesoderm**

A: opposing Gradients of Wnt3a (blue), FGF8 (red) and retinoic acid (green) pattern the anterior-posterior axis of the presomitic mesoderm. B: In the somites, a combination of mediolateral and dorsoventral signals pattern tissue identity, including BMP4 from the lateral plate (blue), Wnt6 from the surface ectoderm (green) and Wnt1 from the dorsal neural tube (yellow), and SHh from the notochord (red; after Dietrich *et al.*, 1997 and Linker *et al.*, 2005).

the pace of segmentation (Gibb *et al.*, 2009), the FGF pathway in the positioning of the determination front (Dubrulle *et al.*, 2001), and the retinoic acid pathway in the cessation of axis extension (Tenin *et al.*, 2010) and left-right synchrony (Morales *et al.*, 2007).

After segmentation, inductive signals from the surrounding tissues are known to be important in patterning the somites (fig. 1.10b). Signals from the notochord and floorplate, such as Sonic Hedgehog (SHh), are important in dorsoventral patterning, required both for the sclerotome (Christ *et al.*, 2004) and the dermomyotome (Maroto *et al.*, 1997), while Wnts and BMPs from the surface ectoderm are important in patterning dorsal and mediolateral identity (Maroto *et al.*, 1997, Reshef *et al.*, 1998, Dietrich *et al.*, 1997). As development proceeds, these signals are also used to specify subregions within the somitic derivatives, such as the differential role of Wnts in patterning the epaxial and hypaxial dermomyotome (Linker *et al.*, 2005), and the action of SHh and BMP in patterning the sclerotome (Christ *et al.*, 2004).

However, with the exception of the interaction of Notch signalling with *MesP* and eph-ephrin signalling, little is known about the role of cell signalling in patterning the rostral PSM. A combination of Wnt1 and SHh signalling is needed to induce *Pax3/7* (Maroto *et al.*, 1997), and Wnt6 signalling is required

for maintenance of *Paraxis* (Linker *et al.*, 2005), but little is known about the regulation of these factors *in vivo*.

# Transcription Factors

The paraxial mesoderm is comprised of a variety of cell lineages, from the primordial mesenchyme of the PSM to its derivatives the sclerotome, myotome and dermatome. These derivatives are also subject to subtle subdivisions, for example the differentiation between hypaxial and epaxial dermomyotome. A complex network of transcription factors regulates the changes in cell identity throughout its development. Transcription factors like *Tbx6* and the various Pax genes are thought to regulate cell identity, while other factors such as *MesP*, *Paraxis* and *Snail* appear to have specific roles in regulating the morphological transitions which paraxial mesoderm undergoes throughout development. Additionally, each lineage is associated with specific transcription factor networks, such as the MRF family in the dermomyotome, which combine to produce the range of subtypes required for mature tissue morphology.

## The Paired-Box Family

*Paired-box* or Pax genes are important regulators in vertebrate development. The vertebrate family of nine Pax genes have been divided into four groups based on their structural properties. They are most prominently associated with the nervous system, although they are also known to be expressed in the neural crest, as well as mesodermal derivatives. Three groups in particular

are associated with the mesoderm, in the dermomyotome (*Pax3/7*), sclerotome (*Pax1/9*), and pronephros (*Pax2/8*).

There are several general features of Pax genes that bear highlighting; they tend to form functionally related pairs (or triplets in the case of *Pax2/5/8*), of which one tends to be dominant, is expressed sooner and has a more profound phenotype, and exhibits less rundundancy than it's partner. Additionally, all Pax genes appear to exhibit a level of dose dependency, and most of them are haploinsufficient.

### *Pax1/9*

The *Pax1/9* group is unique among the Pax family, in that they are not expressed in the nervous system. *Pax1* is detected in the developing axial skeleton, (Deutsch *et al.*, 1988) limb buds (Timmons *et al.*, 1994), pharyngeal pouches and thymus (Wallin *et al.*, 1996). *Pax9* has a similar pattern of expression (Neubuser *et al.*, 1995). However, while it is not expressed in the neural tube, mutations in *Pax1* are thought to contribute to some forms of congenital spina bifida in humans (Joosten *et al.*, 2005).

A spontaneous mutation in *Pax1* produced the *undulated* allele (Wright, 1947), which was characterised by defects in the axial skeleton (Grüneberg, 1950, Grüneberg, 1954). Two more severe mutants were shown to be allelic to *undulated*, *short-tail* (Blandova and Egorov, 1975) and *undulated-extensive* (Wallace, 1985). The mutation causing *undulated* was identified as a single

amino-acid substitution in the paired domain of *Pax1* (Balling *et al.*, 1988), with the more severe alleles identified as large deletions (Dietrich and Gruss, 1995; Wallin *et al.*, 1994). A true null mutant was generated in 1998 (Wilm *et al.*, 1998), confirming a heterozygous phenotype and suggesting that the function of Pax1 in some lineages is haploinsufficient. In contrast, *Pax9* mutants exhibit no obvious defects of the axial skeleton, although they do show defects of the skeleton of the limb and skull (Peters *et al.*, 1998); however *Pax1/Pax9* double knockouts have an extremely severe vertebral phenotype, suggesting functional redundancy between these two genes in the formation of the axial skeleton (Peters *et al.*, 1999).

In the paraxial mesoderm, *Pax1/9* is associated with the sclerotome. Activation of *Pax1* is dependent on *Paraxis*, a bHLH transcription factor in the rostral PSM required for epithelialisation (Wilson-Rawls *et al.*, 2004). *Pax1* is first expressed in the epithelial somite, and thus precedes EMT, while *Pax9* is not expressed until the sclerotomal mesenchyme has formed (Muller *et al.*, 1996). However, two subpopulations of cells at the dorsal and lateral extents of the sclerotome, which give rise to the ribs and neural arches, are *Pax1* negative (Ebensperger *et al.*, 1995). This reflects the fact that strong ventromedial signals from the notochord and floorplate appear to be required for the expression and maintenance of *Pax1*, chiefly SHh and Noggin (reviewed in Christ *et al.*, 2004), in competition with signals from the dorsal and lateral embryo. In contrast to the dermomyotome, while ventral cells undergo EMT rapidly after segmentation, they are not committed to a

sclerotomal fate and require ventromedial signals to maintain both *Pax1* and sclerotome identity (Dockter and Ordahl, 2000). *Pax9* initially exhibits a similar expression pattern to *Pax1*, but as development progresses they become segregated to medial and lateral domains of the central sclerotome respectively (Peters *et al.*, 1995). As the sclerotome resegments, *Pax1* is downregulated in the vertebral body, becoming restricted to the intervertebral discs and perichondrial cells. *Pax1* appears to be required for the correct formation of ventral vertebral structures, such as the vertebral bodies, while dorsal and lateral structures (neural arches and ribs respectively), whose precursors do not express *Pax1*, develop almost normally (Wallin *et al.*, 1994).

The cellular function of *Pax1/9* in the sclerotome is not clear, but they appear to regulate patterning; for example in *Pax1/Pax9* double-null embryos, the sclerotome fails to undergo chondrogenesis. However, the sclerotome also exhibits a deficiency in proliferation, and a later increase in apoptosis, suggesting that *Pax1/9* may also have a role in proliferation and survival (Peters *et al.*, 1999).

### *Pax2/8*

*Pax2*, the founding member of the *Pax2/5/8* family, is predominantly associated with the development of the urogenital system (Dressler *et al.*, 1990), although it is also expressed in the nervous system (Nornes *et al.*, 1990). It is first upregulated in the intermediate mesoderm, where along with

*Pax8* it regulates the morphogenesis of the pronephros (Plachov *et al.*, 1990, Bouchard *et al.*, 2002). The third member of the family, *Pax5*, is involved in brain patterning and is required for the B-cell lineage in the immune system, but is not involved in mesoderm patterning (Urbanek *et al.*, 1994). The *Pax2* knockout phenotype is primarily associated with the urogenital system, with homozygotes completely lacking kidney, bladder, or genitals (Torres *et al.*, 1995). Consistent with the dose dependency observed in other Pax genes, *Pax2* is haploinsufficient, and heterozygotes exhibit hypomorphic kidneys. *In vitro* studies suggest that one function is to mediate a mesenchyme-to-epithelial transition in this tissue (Rothenpieler and Dressler, 1993).

Intriguingly, it has been observed in chickens that *Pax2* is expressed in the rostral PSM, and exhibits cyclic activation similar to *EphA4* (Suetsugu *et al.*, 2002). Furthermore, gene activation studies have shown that *Pax2* is activated by, and mediates part of the function of, *Meso1* in the paraxial mesoderm (Watanabe *et al.*, 2007). Unlike *EphA4*, however, which is also a target of *Meso1*, *Pax2* cannot drive boundary formation (Watanabe *et al.*, 2009), suggesting that *Pax2* mediates a separate aspect of *Meso1* function. Interestingly, *Pax2* has not been detected in the PSM of mice, and may represent a species difference in function (Suetsugu *et al.*, 2002).

### *Pax3/7*

*Pax3* and *Pax7* form a subfamily of Pax genes primarily associated with dorsal neural tube patterning and the formation of the musculature

(Buckingham and Relaix, 2007). *Pax3* has also been implicated in a number of other lineages, including neural crest derivatives such as melanocytes and populations in the urogenital system and colonic epithelium (Engleka *et al.*, 2005), although in the case of the urogenital system, this probably represents a contribution of cells from the paraxial mesoderm to the stromal cells of the kidneys rather than expression of *Pax3* in the intermediate mesoderm (Guillaume *et al.*, 2009).

## Knockout Mutants

As with several Pax genes, a spontaneous mutation of *Pax3* was identified in the pre-genomic era (Auerbach, 1954). Because of haploinsufficiency of *Pax3* in the melanocyte lineage of the neural crest (Conway *et al.*, 1997), the heterozygous mice had characteristic white patches on their belly, limbs and faces, leading to the line being christened *splotch*. More severe alleles were generated using mutagenesis screens, including *splotch-delayed*, *splotch2H*, and *splotch-retarded*. The original splotch mutation was identified as a single amino acid change in a splice acceptor site of *Pax3* (Epstein *et al.*, 1993; fig 1.11b), while the more severe alleles consisted of larger deletions, with *splotch-retarded* constituting a large scale chromosomal deletion (Epstein *et al.*, 1991a). Of particular note is *splotch2H*, which was considered to be closest spontaneous mutant to a *Pax3*-null mutation (Epstein *et al.*, 1991b; fig. 1.11d). This was ultimately confirmed by the generation of a targeted *Pax3* knockout (Mansouri *et al.*, 2001; fig. 1.11e).

The primary phenotype of *splotch* homozygotes are neural tube closure defects, spina bifida and exencephaly, which cause perinatal death (Auerbach, 1954). However, a portion of homozygotes die during development due to cardiac malformations (Conway *et al.*, 1997). Homozygotes also exhibit a profound muscular phenotype, with a complete absence of muscles of the limbs (Franz *et al.*, 1993) tongue (Zhou *et al.*, 2008) and diaphragm (Li *et al.*, 1999), cells which originate from a population of long-range migratory cells in the hypaxial dermomyotome (reviewed by Buckingham *et al.*, 2003). These defects are caused by apoptosis in the dermomyotome, especially the hypaxial lip of the dermomyotome which exhibits the strongest expression of *Pax3* (Goulding *et al.*, 1994) and is severely affected or even completely lost in *Pax3* mutants (Borycki *et al.*, 1999). The epaxial lip of the dermomyotome, which also expresses *Pax3*, is also affected to a lesser extent in mutants. The cell-intrinsic nature of this effect was confirmed using *Pax3*-null chimeras, showing that the effect on the dermomyotome was independent of effects on other tissues such as the dorsal neural tube and neural crest (Mansouri *et al.*, 2001). There is also evidence that *Pax3* mutations cause defects in the segmental patterning sclerotome, raising the possibility that it is involved at an early stage in segmentation (Schubert *et al.*, 2001).

In contrast, *Pax7* mutants have an extremely mild phenotype (Mansouri *et al.*, 1996), despite a very similar expression pattern in the embryo (Jostes *et al.*,

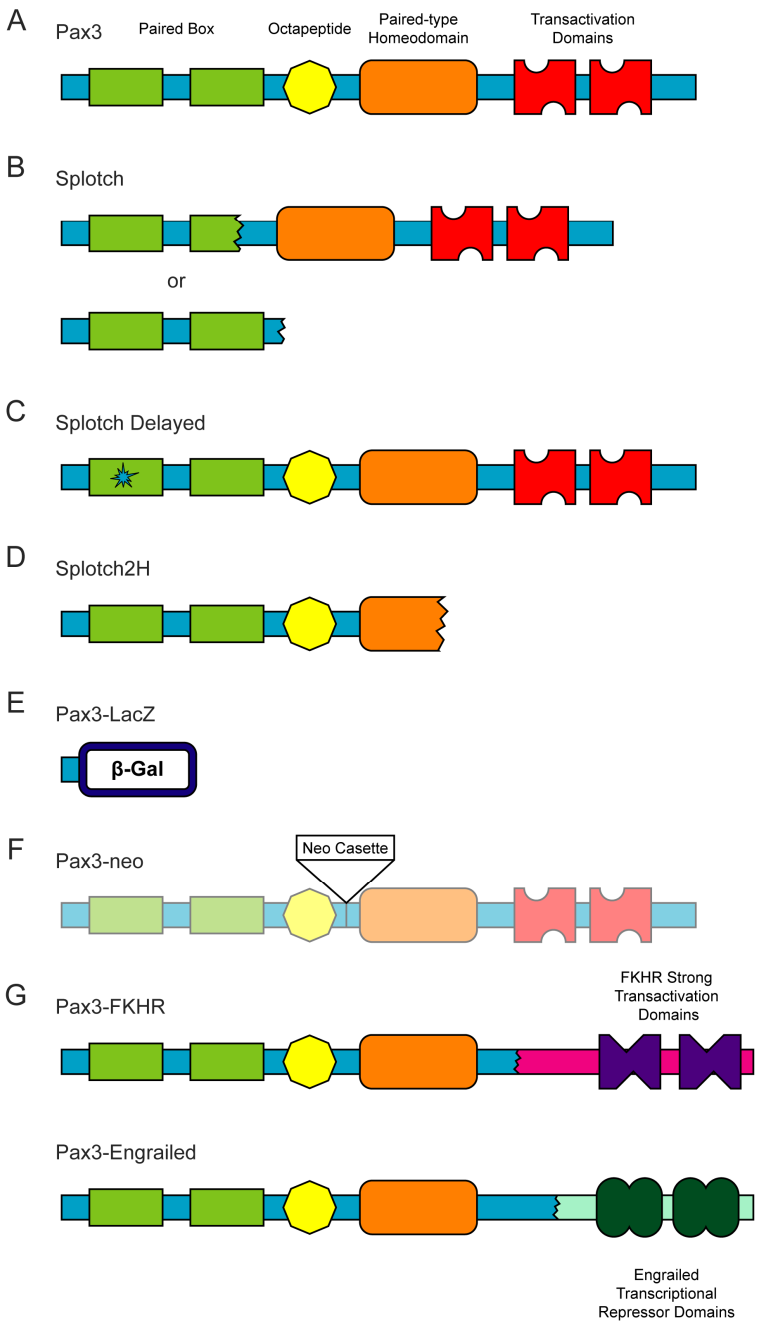


**Figure 1.11: *Pax3* Mutants**

Mutant mice have been used widely to understand the function of *Pax3*. A: wild type Pax3 protein structure. B: naturally occuring s*plotch* mutants contain a point mutation in a splice acceptor site, producing a mixture of two possible protein forms. C: *Splotch delayed* is mild mutant caused by a point mutation in Paired domain. D: *Splotch2H* is an artificially generated mutant producing a truncated protein. E: transgenic knockout replacing *Pax3* locus with *LacZ*. F: hypomorphic *Pax3* allele produced by inclusion of a thymidine kinase-neomycin resistance cassette, producing wild-type protein at approximately 20% of wild type levels (Zhou *et al.*, 2008). G: *Pax3* fusion proteins Pax3-FKHR (Relaix *et al.*, 2003) and Pax3-Engrailed (Relaix *et al.*, 2005). (A-E after Greene *et al.*, 2009).

David Wright

1990). Homozygous mutants survive until birth, with only very minor defects in facial morphology (possibly due to neural crest defects), however postnatal growth is retarded and pups die within three weeks. This is due to a loss of satellite cells, which function as adult stem cells for postnatal skeletal muscle (Seale *et al.*, 2000). Later observations showed that satellite cells develop correctly, but are unable to survive without *Pax7* (Relaix *et al.*, 2005).

In *Pax3/Pax7* double knockouts, the majority of the trunk musculature is absent, with only a small population of *Pax*-independent *Myf5*-expressing cells generating an early myotome (Relaix *et al.*, 2005). This suggests that *Pax7* can compensate for *Pax3* in the central and epaxial dermomyotome, while *Pax3* can functionally replace *Pax7* throughout the dermomyotome. The hypaxial domain of the dermomyotome, which is defective in *Pax3* mutants, does not express *Pax7*. *Pax7* is sufficiently similar to *Pax3* that when it is inserted into the *Pax3* locus, it can rescue almost all of the *Pax3*-null phenotype, including neural defects and hypaxial trunk musculature, but not the long-range migrating lineages (Relaix *et al.*, 2004), possibly due to being unable to upregulate c-Met in the hypaxial dermomyotome. Interestingly, the authors note that *Pax7* is capable of transactivating c-Met *in vitro*, and suggest that the difference may be due to an absence of *Pax7*-specific coactivators in the hypaxial lineage. Interestingly, dermomyotome cells lacking both *Pax3* and *Pax7* do not all undergo apoptosis; some were detected as Collagen2a-positive bone progenitors. *Pax7* expression appears to be negatively regulated by *Pax3*, as in *splotch* mutants its expression

extends into *Pax3* domains in the neural tube and dermomyotome (Borycki *et al.*, 1999). However, another report has suggested that *Pax7* is downregulated in *splotch* mutant lysates (Zhou *et al.*, 2008).

Because the hypaxial dermomyotome is most severely affected in *Pax3* mutants, a lot of research has been focussed on how *Pax3* affects this lineage specifically. It is known to regulate differentiation by promoting the muscle differentiation factor *MyoD* (Maroto *et al.*, 1997; Tajbakhsh *et al.*, 1997), however, presumptive hypaxial dermomyotome cells from *splotch* mice grafted into the limb buds of chicken embryos correctly differentiate into myoblasts (Daston *et al.*, 1996). *Pax3* may also be required for migration, as it regulates c-Met, a receptor required for delamination and migration from the hypaxial dermomyotome (Bladt *et al.*, 1995). Even in the neural crest, which does migrate in the absence of *Pax3*, migratory potential is altered, suggesting a possible role in persistance or cell guidance (Epstein *et al.*, 2000). However, *Pax3* is also an important regulator of cell survival in the hypaxial dermomyotome (Borycki *et al.*, 1999).

## Hypomorphs, Fusion Proteins and Dose Dependance

A general feature of Pax genes appears to be dosage dependence, illustrated by haploinsufficient mutant phenotypes. In reporter studies, it has been shown that low levels *Pax3* can upregulate the transcriptional activity of a Pax binding domain while high levels repress activity (Chalepakis et al 1994). The

importance of dosage *in vivo* has been investigated using hypomorphic mutants and gain-of-function fusion proteins.

In attempting to generate a conditional knockout of the *Pax3* homeodomain (Koushik *et al.*, 2002), researchers noticed that an intermediate transgenic line containing a neomycin-resistance casette between exons 5 and 6 exhibited a hypomorphic phenotype (*Pax3-neo*; Zhou *et al.*, 2008; fig. 1.11f). The precise cause of this effect is not clear, but has been observed in several cases (Meyers *et al.*, 1998; Koni *et al.*, 2001; Holzenberger *et al.*, 2000), also observed in the *Pax3-FKHR* transgene created by Lagutina *et al.*, 2002, and may be as a result of aberrant mRNA splicing. The net effect was an allele which uniformly produced wild-type Pax3 protein at one fifth of normal levels, allowing the study of the effect of varying the dosage of *Pax3*.

*Pax3-neo* hypomorphic embryos had normal diaphragm muscle and were viable at birth, but exhited dysmorphic limbs and a lack of tongue musculature, and died postnatally due to an inability to feed. This suggested a striking correlation between long-range hypaxial migration (i.e. to the limbs and tongue) and dosage of *Pax3*, with short-range hypaxial migration exhibiting a lower threshold dependence on *Pax3*. Interestingly, the defect appeared to be due to apoptosis in the hypaxial dermomyotome, raising the possibility that a subpopulation of myoblasts with different migratory potentials are specified by *Pax3* dosage.

Intriguingly, *Pax7* is known to be expressed in short-range migratory myoblasts (Gross *et al.*, 2000). The observation that *Pax3* hypomorphs express elevated *Pax7*, while *Pax3* knockouts show a downregulation (Zhou *et al.*, 2008), suggests that the presence of *Pax7* may be involved in the short-range migration phenotype. A comparable effect was observed in mice expressing *Pax7* in the *Pax3* locus, where short-range migration of the trunk musculature was rescued, but long range migration to the limbs was not (Relaix *et al.*, 2004).

Interestingly, in both the *Pax3-neo* hypomorph and *Pax7* knockin, a small population of myoblasts were detected in the hindlimb bud (Relaix *et al.*, 2004; Zhou *et al.*, 2008), raising the possibility that the requirement for *Pax3*, or potentially the inability of *Pax7* to substitute for *Pax3* in the hypaxial dermomyotome, is less pronounced in the caudal embryo.

Transcription factors may regulate gene expression positively or negatively depending on cellular context, and *Pax3* has been shown to both activate and repress gene expression (Khan *et al.*, 1999; Mayanil *et al.*, 2001). In order to dissect the role of activation or repression in Pax3 function, two transgenic forms of *Pax3* have been generated by fusing transcriptional activation or repression domains to the *Pax3* protein.

*Pax3*- and *Pax7-FKHR* fusions arise spontaneously by translocation of the *Forkhead box O1* (*FOXO1*, previously known as *FKHR*) transactivation

domain into the *Pax3* or *Pax7* locus (fig. 1.11g). Both fusions give rise to a childhood sarcoma, Alveolar Rhabdomyosarcoma (Galili *et al.*, 1993; Davis *et al.*, 1994). The *Pax3-FKHR* fusion is thought to activate transcription at all *Pax3* binding sites. For example, it has been shown to increase transcription by about 100-fold (Bennicelli *et al.*, 1996), but there is also evidence that it transforms transcriptional inhibition into activation (Cao and Wang, 2000), activating gene targets that are not normally regulated by (and may even be inhibited by) *Pax3* (Epstein *et al.*, 1998).

Two *Pax3-FKHR* transgenic mouse lines were independently created (Lagutina *et al.*, 2002; Relaix *et al.*, 2003), however one exhibited a low expression rate potentially due to the inclusion of a neomycin resistance casette (Lagutina *et al.*, 2002). While there were differences between the mutants, both exhibited neonatal lethality due to respiratory failure, caused by abnormalities of the diaphragm (Lagutina *et al.*, 2002) or ribs (Relaix *et al.*, 2003). However, *Pax3-FKHR* was able to rescue aspects of the *Pax3* knockout phenotype, including limb musculature and spina bifida/exencephaly. This suggests that transcriptional activation is the primary function of *Pax3* in myogenesis, neural tube closure and neural crest formation (Relaix *et al.*, 2003).

Since the *Pax3-FKHR* is a stronger transcriptional activator than *Pax3* (Fredericks *et al.*, 1995; Bennicelli *et al.*, 1996), it is possible to consider *Pax3-FKHR* a gain-of-function mutant (although it should also be remembered

that it may also convert any inhibitory activity of wild type *Pax3* into activation). From this perspective, the defects in proximal muscles such as the diaphragm and rescue of distal muscle defects neatly inverts the loss of long-distance migrating muscles in the *Pax3-neo* hypomorph (Zhou *et al.*, 2008).

However, some aspects of transcriptional regulation are aberrant in *Pax3-FKHR* mice, especially regulation of c-Met. This receptor is ectopically expressed in the *Pax3*-positive domain of the neural tube, although not in the *Pax3*-positive domains of the facial primordia. In the hypaxial dermomyotome, expression of *c-Met* is massively upregulated, and drives ligand-independent signalling and consequently ectopic delamination and migration (Relaix *et al.*, 2003).

In an attempt to investigate transcriptional repression by *Pax3*, Bajard and colleagues generated a transgenic line containing a *Pax3-Engrailed* fusion protein (Bajard *et al.*, 2006; fig. 1.11g). *Engrailed* is a Drosophila multifunctional transcription factor containing activation and repression domains (Morgan, 2006). When the C-terminal transcriptional repressor domain of *Engrailed* is fused to a DNA-binding protein, the transgene can efficiently silence transcription (Han and Manley, 1993, John *et al.*, 1995). *Pax3* and *Pax7* fusion proteins containing the *Engrailed* repressor domain were shown to be effective at inhibiting *Pax3/7* dependent transcription in vitro (Relaix *et al.*, 2006). In *Pax3-Engrailed* heterozygous transgenic mice, *Pax3* and *Pax3-Engrailed* are thought to compete for DNA binding sites, producing

a hypomorphic phenotype. The *Pax3-Engrailed* mutant appears to be a *Pax3* hypomorph, but crucially retains the hypaxial dermomyotome, which is lost by apoptosis in all other *Pax3* loss-of-function mutants, including the *Pax3-neo* hypomorph (Zhou et al, 2008). However, as with the *Pax3-neo* mutant, long-range migration of myoblasts was impaired, with only a few cells detected in the hindlimb and tongue, and none in the forelimb.

In summary, while simple knockout mutants of *Pax3* showed deficiency in almost all body musculature, the array of hypomorphic, gain- and loss-of-function mutants reveals that the dose of *Pax3* seems to mediate migratory potential, with high-*Pax3* expressing cells migrating long distances to populate the limbs and tongue, while lower-*Pax3* expressing cells travel short distances to form the diaphragm and intercostal muscles.

## *Pax3* in Segmentation

The role of *Pax3/7* in the derivatives of the paraxial mesoderm has generally assumed to be limited to its role in myogenesis. However, it has been shown that *Pax3* is expressed in the whole somite during segmentation (Goulding *et al.*, 1994), and that *Pax3* mutations give rise to defects in the axial skeleton (Henderson *et al.*, 1999). This was initially attributed to the loss of the hypaxial dermomyotome and consequent disruption of signalling to the lateral sclerotome, however a closer investigation revealed a role for *Pax3* in segmental patterning, with disrupted anteroposterior patterning and altered expression of *EphA4* (Schubert *et al.*, 2001) but readouts of the segmentation

clock such as *Lfng* showed normal oscillations in the PSM. Expression of *Paraxis* is altered in *Pax3* mutant embryos (Henderson *et al.*, 1999), although *Paraxis* appears normal in the PSM and early somites. Conversely, *Pax3* appears to be disrupted in *Paraxis* mutants (Burgess *et al.*, 1995), which show similar anteroposterior disruptions (Johnson *et al.*, 2001). It has also been observed that *Pax3* interacts with members of the T-box family in vitro, such as the anterior somite marker *Tbx18* (Farin *et al.*, 2008). Mice deficient in both *Tbx18* and *Pax3* have more severe anteroposterior patterning defects.

It has been suggested that *Pax3* may also function in the epithelialisation of the somite. Surgical manipulations designed to elucidate the signalling pathways controlling formation of the dermomyotome (Dietrich *et al.*, 1997, Dietrich *et al.*, 1998) hinted at a role of *Pax3* in formation or maintenance of an epithelium; wherever *Pax3* was induced or maintained, the somitic epithelium remained in place. It was also shown that transfection with *Pax3* was sufficient to produce a mesenchyme-to-epithelial transition in cell culture (Wiggan *et al.*, 2002). While the dermomyotome loses it's epithelial cohesion in *Pax3* mutants (Mansouri *et al.*, 2001), an epithelial somite is transiently present in the absence of *Pax3*; this may be due to redundancy with other factors such as *Pax7* and *Paraxis*.

## Targets of *Pax3*

Myogenesis is controlled by a complex network of transcription factors. It was initially thought that *Pax3* and *Myf5* operated as parallel pathways to control

*MyoD* (Tajbakhsh *et al.*, 1997), as the *Pax3-FKHR* gain-of-function mutant exhibited an upregulation of *MyoD* without *Myf5* (Relaix *et al.*, 2003). However, *in vitro* assays in the chick suggested that *Pax3* may activate *Myf5* (Maroto *et al.*, 1997). This was confirmed *in vivo* by the discovery that the hypaxial domain of *Myf5* is lost in *Pax3-Engrailed* hypomorphs, and that *Pax3* binds to and transactivates the promoter of *Myf5* (Bajard *et al.*, 2006). Interestingly, another layer of control was observed when studying the role of Wnt signalling on the dermomyotome: the transcriptional activity of *Pax3* seems to be modulated by the PKC-dependent non-canonical Wnt signalling pathway without affecting the underlying expression of *Pax3* (Brunelli *et al.*, 2007).

An important target of *Pax3* is c-Met, the receptor for scatter factor/hepatocyte growth factor. This receptor is expressed in the hypaxial and epaxial dermomyotome, and is required for delamination and migration of myoblasts to the limb buds (Bladt *et al.*, 1995). It has been shown to be regulated by *Pax3* in vitro (Wiggan *et al.*, 2002) and in vivo (Epstein *et al.*, 1996).

*Pax3* has also been associated with regulation of the Ig-like superfamily cell adhesion molecule NCAM. It is expressed in a similar pattern to *Pax3* (Goulding *et al.*, 1991, Edelman, 1986), and even before the characterisation of the *splotch* allele as *Pax3*, differences in NCAM expression had been observed in *splotch* mutants (Morris and O'Shea, 1983; Moase and Trasler, 1991), suggesting a qualitative difference in the NCAM expression profile in

the absence of *Pax3*. Interestingly, while *Pax3* does not bind to the *NCAM* promoter *in vitro*, the truncated *splotch* isoform does (Chalepakis *et al.*, 1994), explaining some gain-of-function effects in the *splotch* mutant.

*Pax3* may also modulate the function of NCAM by controlling its glycosylation state. Microarray and *in vitro* studies have shown that *Pax3* can directly upregulate an enzyme involved in NCAM glycosylation (Mayanil *et al.*, 2001). However, *Pax3* mutants have been described to affect the polysialylation of NCAM in contradictory ways (Neale and Trasler, 1994; Glogarova and Buckiova, 2004).

## The Basic Helix-Loop-Helix Family

### *Paraxis*

The Twist subfamily of bHLH transcription factors, many of which are involved in the mesodermal lineage, includes the *Paraxis* transcription factor (Barnes and Firulli, 2009). It's early expression is concommitant with gastrulation, detected in the primitive mesoderm of mice at E7.5 (Burgess *et al.*, 1995). In chicken it is first detected in the mesoderm adjacent to the node at full extension of the primitive streak (HH4; Barnes *et al.*, 1997) in the region where the first somite will form. At later stages, it is expressed in the rostral paraxial mesoderm, and is maintained in the somites. As the somites mature, it is downregulated in the sclerotome (Burgess *et al.*, 1995), and is ultimately downregulated in the paraxial mesoderm by E13.5; although it is also

detectable in the limb buds and adult muscle tissue (Delfini and Duprez, 2000; Burgess *et al.*, 1995).

The role of *Paraxis* in somitogenesis was underscored by its unusual mutant phenotype. Mutant embryos failed to form epithelial somites, despite correct segmental patterning and gap formation (Burgess *et al.*, 1996), indicating that this factor is required for epithelialisation of somites. However, some defects in anterior-posterior somite patterning have been observed which may represent more than a failure to form discrete segmental units, for example while the Notch ligand *Delta1* is expressed normally in the PSM, it is lost in the caudal somite, and the segment polarity marker *EphrinB2* is diffuse in newly formed somites (Johnson *et al.*, 2001). However, the posterior segment polarity marker *Uncx4.1* is correctly expressed, suggesting that some segment polarity function is still present (Takahashi *et al.*, 2007). *EphA4* is associated with boundary formation in segmentation, raising the possibility that aberrant eph-ephrin signalling may contribute to the *Paraxis* mutant phenotype, however *EphA4* expression appears to be normal and *EphrinB2* is expressed in an appropriate domain in the rostral PSM (Johnson *et al.*, 2001). Experiments in the chick demonstrated that *Paraxis* could promote incorporation into the somitic epithelium using a mechanism that required the small GTPase *Rac1* (Nakaya *et al.*, 2004).

Mutant embryos were viable, demonstrating that *Paraxis* was not required for the differentiation of somite-derived tissue such as muscle, skeleton and

dermis; however there were gross abnormalities consistent with the disruption of cell organisation within segments, such as fused vertebrae and intercostal muscles (Burgess *et al.*, 1996, Wilson-Rawls *et al.*, 1999). Similarily, the patterns of many differentiation markers, such as *Pax3/7* in the dermomyotome and especially *Pax1/9* in the sclerotome, are disrupted (Takahashi *et al.*, 2007). A role for *Paraxis* in controlling proliferation has also been suggested, as *Paraxis* mutants incorporate less BrdU (Wilson-Rawls *et al.*, 1999), which is consistent with general hypotrophy in the mutant musculature.

However, a more subtle role for *Paraxis* in myogenic patterning has been suggested. In *Paraxis* mutants, while *Pax3* expression is normal in the PSM and early somites, it is lost in the maturing somites and dermomyotome. A much reduced population of *Paraxis*-independent *Pax3*-positive cells are detectable in the hypaxial dermomyotome at later stages (Wilson-Rawls *et al.*, 1999). *Paraxis/Myf5* double knockouts show a complete lack of epaxial muscles, which are present in both *Myf5* and *Paraxis* mutants. In *Myf5* mutants, a population of *Pax3/MyoD* expressing cells are thought to compensate for loss of *Myf5*, and this compensation appears to be dependent on *Pax3*. Interestingly, *Pax3* may also be required for maintenance of *Paraxis* in the late somite (Henderson *et al.*, 1999), underscoring the complexity of reciprocal genetic networks in the myogenic lineage.

The role of *Paraxis* in the maturing somites was dissected more finely using

microsurgical techniques in the chick embryo (Linker *et al.*, 2005). It was shown that signals from the ectoderm (but not lateral plate or neural tube) were required to maintain *Paraxis* and the epithelial patterning of the dermomyotome. These signals were identified as Wnts, especially Wnt6 acting through the canonical β-catenin pathway. Wnt6 has also been described to drive ectopic myogenesis in the limb bud via *Pax3* and *Myf5* (Geetha-Loganathan *et al.*, 2005). However, it was not convincingly shown that ectodermal signals are required for the initial induction of *Paraxis* in the rostral PSM; indeed, the expression of Wnt6 target *Frizzled-7* in the rostral PSM are not consistent with a role in regulating *Paraxis* (Linker *et al.*, 2005).

### *Mesoderm Posterior (MesP)*

Another family of bHLH transcription factors involved in segmentation are *MesP1* (Saga *et al.*, 1996) and *MesP2* (Saga *et al.*, 1997), also called *Meso1* (Buchberger *et al.*, 1998) and *Meso2* (Buchberger *et al.*, 2002) in chicken (not to be confused with mouse *Meso1*, an early name for *Paraxis*; Blanar *et al.*, 1995).

*MesP1* was first identified in a differential screen designed to identify factors involved in primordial germ cell differentiation, which occurs at gastrulation (Saga *et al.*, 1996). Both *MesP1* and its chick orthologue *Meso1* are strongly expressed in the nascent mesoderm as it emerges from the primitive streak (Saga *et al.*, 1996; Buchberger *et al.*, 1998), however this domain of

expression is restricted to the tailbud and subsequently lost as development proceeds. *MesP1* is also expressed in the rostral PSM (Saga *et al.*, 1997).

During the isolation of *MesP1*, a second gene was identified with a nearly identical bHLH domain, and closely related on the same chromosome (Saga *et al.*, 1997), and named *MesP2*. This gene lacked the expression domain in the nascent mesoderm, but showed an identical expression pattern in the rostral PSM to *MesP1* (Saga *et al.*, 1997). A chicken orthologue, *Meso2*, was also identified with similar homology, close chromosomal position, and expression pattern to *Meso1* (Buchberger *et al.*, 2002). However, the expression pattern in *Meso2* showed a subtle variation from *Meso1*, with *Meso2* transcripts persisting longer than *Meso1*. Intriguingly, while a LacZ reporter showed that in the mouse *MesP* is briefly expressed throughout the full extent of the presumptive somite before being localised to the anterior compartment (Takahashi *et al.*, 2000), in the chick *Meso* is localised to the posterior compartment (Buchberger *et al.*, 2002).

## Mutants

Mice lacking *MesP1* had severe defects and died by E10.5 (Saga, 1998). However, embryos appeared to produce normal axial tissues and PSM, and while segments were hypotrophic and showed patterning defects, definitive somite boundaries were present. In contrast, *MesP2* knockout mice failed to form segmental borders or epithelial somites, although an aberrantly segmented epithelial dermomyotome did form, and myogenic markers were

detected (Saga *et al.*, 1997). Consequently, the sclerotome of *MesP2*-null mice loses segmental patterning, and results in a complete fusion of the vertebral column. Furthermore, this appeared to represent an expansion of caudal somite derivatives at the expense of rostral; both in terms of skeletal structures and aberrant patterning of the dorsal root ganglion neurites, which migrate through the rostral compartment of the sclerotome (Saga *et al.*, 1997). This observation was strikingly confirmed using genetic markers of somite polarity; in *MesP2* knockout embryos, the rostral somite markers *Delta1* and *EphA4* are lost and caudal marker *Uncx4.1* is expanded (Takahashi *et al.*, 2000, Nomura-Kitabayashi *et al.*, 2002). *MesP2* is also required for the rapid degradation of *Tbx6* protein at the PSM-Somite boundary, and despite correct downregulation at the mRNA level, in *MesP2* mutants *Tbx6* protein is maintained into the somitic domain of the paraxial mesoderm (Oginuma *et al.*, 2008), which could explain part of the *MesP2* phenotype.

Interestingly, *MesP2* is capable of rescuing the *MesP1*-null phenotype when it is inserted into the *MesP1* locus (Saga, 1998), raising the possibility of functional redundancy between the two genes. Indeed, when the *MesP1*-null embryo was examined, ectopic *MesP2* was detected in the nascent mesoderm, and a *MesP1/MesP2* double knockout resulted in a complete absence of non-axial mesoderm, suggesting that *Mesp2* can functionally compensate for *Mesp1* (Kitajima *et al.*, 2000). On the other hand, *Mesp1* expression was normal in *Mesp2* mutant embryos (Saga *et al.*, 1997),

suggesting that *Mesp1* cannot functionally compensate for *Mesp2* in segmental patterning.

## Interactions with other Segmental Genes

Defective epithelialisation and segmental polarity raised the possibility that *MesP2* and *Paraxis* may cooperate in segmentation. The genes do not appear to directly regulate each other, as *MesP2* expression is normal in *Paraxis* mutants (Johnson *et al.*, 2001), and vice versa (Saga *et al.*, 1997). Researchers did not find evidence of a direct molecular interaction between the two factors, but found that double mutants had an exacerbated phenotype (Takahashi *et al.*, 2007). Particularly, the mild sclerotomal defects caused by loss of *Pax1* in the *Paraxis* mutant was exacerbated in *MesP2/Paraxis* double mutants by the loss of *Pax9*, which results in a profound loss of axial skeleton similar to the *Pax1/9* double knockout phenotype (Peters *et al.*, 1999). Interestingly, while *Pax3* is expressed in the anterior PSM of both *Paraxis* and *MesP2* mutants, this expression pattern is greatly reduced in the *Paraxis/MesP2* double knockout, suggesting that *Pax3* may be regulated by these genes (Takahashi *et al.*, 2007).

Transfection of *Meso1* into the chick embryo PSM produced an upregulation of NCAM and *Pax2* (Watanabe *et al.*, 2007), as well as *EphA4* (Watanabe *et al.*, 2009). Interestingly, *Pax2* appears to be required for upregulation of NCAM, suggesting that it may mediate and important aspect of *Meso1* function in chick. The role of NCAM is unclear, but appeared to induce

aggregation of cells (Watanabe *et al.*, 2007). This role in cell aggregation is supported by chimeric embryos, where cells that lack *MesP2* are segregated from wild type cells, suggesting a role in cell sorting between adjacent segments (Takahashi *et al.*, 2005), however the role of NCAM in this process has not been investigated. The connection between *MesP* and *Pax2* is unlikely to exist in mice, as these species lack *Pax2* expression in the PSM (Suetsugu *et al.*, 2002).

Interestingly, transfection of *Meso1* also induces the formation of an ectopic border (Watabanbe et al 2009) in a similar way to NICD (Sato et al 2002; see fig. 1.8). This appears to be via upregulation of *EphA4*, which activates *EphrinB2* reverse signalling in an adjacent, non-*Meso1* expressing domain (Watanabe *et al.*, 2009). The connection between *MesP* and *EphA4* has been confirmed in mouse. *MesP1* knockout mice (which do segment) express a reduced level of *EphA4* in the correct domain (Takahashi *et al.*, 2005), while *MesP2* knockouts do not express any *EphA4* (Nomura-Kitabayashi *et al.*, 2002). A *MesP2* hypomorphic allele was found to disrupt segment polarity without disrupting somite boundaries; interestingly the hypomorph maintained *EphA4* expression, further supporting the idea that defective eph-ephrin signalling mediates the *MesP2* segmentation phenotype (Nomura-Kitabayashi *et al.*, 2002). Finally, when the *EphA4* promoter was analysed, it contains binding sites for *MesP2* which strongly activate it's transcription in vivo and in vitro (Nakajima *et al.*, 2006).

## Regulation of *MesP*

Careful observations of *Meso2* expression in the chick revealed that MesP genes are temporally regulated by the segmentation clock (Buchberger *et al.*, 2002). Rather than progressing steadily down the PSM, the expression is transiently expressed in the second presumptive somite (S-1), and this expression is downregulated before (or in the case of *Meso2*, shortly after) the next clock cycle. This was confirmed in mouse by comparing the expression of *MesP2* to the spatial distribution of NICD (Oginuma *et al.*, 2008), a marker of activated Notch signalling and a direct readout of segmentation clock phase (Palmeirim *et al.*, 1997). They showed that *MesP2* is upregulated in phase 2 of the clock cycle, as the wave of Notch signalling passes over the presumptive somite (fig. 1.12). It has been shown that *MesP2* is potently regulated by activated Notch signalling and the PSM marker *Tbx6* (Yasuhiko *et al.*, 2008), which combined with the ability of *MesP2* to downregulate *Tbx6* creates an interesting model where the posterior boundary of *MesP2* domain defines the anterior boundary of the *MesP2* domain for the next cycle (Oginuma *et al.*, 2008; fig. 1.12c). It has been suggested that this boundary may be regulated by FGF signalling, potentially connecting it to the so-called determination front where presumptive somite boundaries are defined in the mid-PSM.

*MesP2* also exerts an effect on Notch signalling. The rostral domain where *MesP2* is upregulated is characterised by slowing of Notch oscillations.

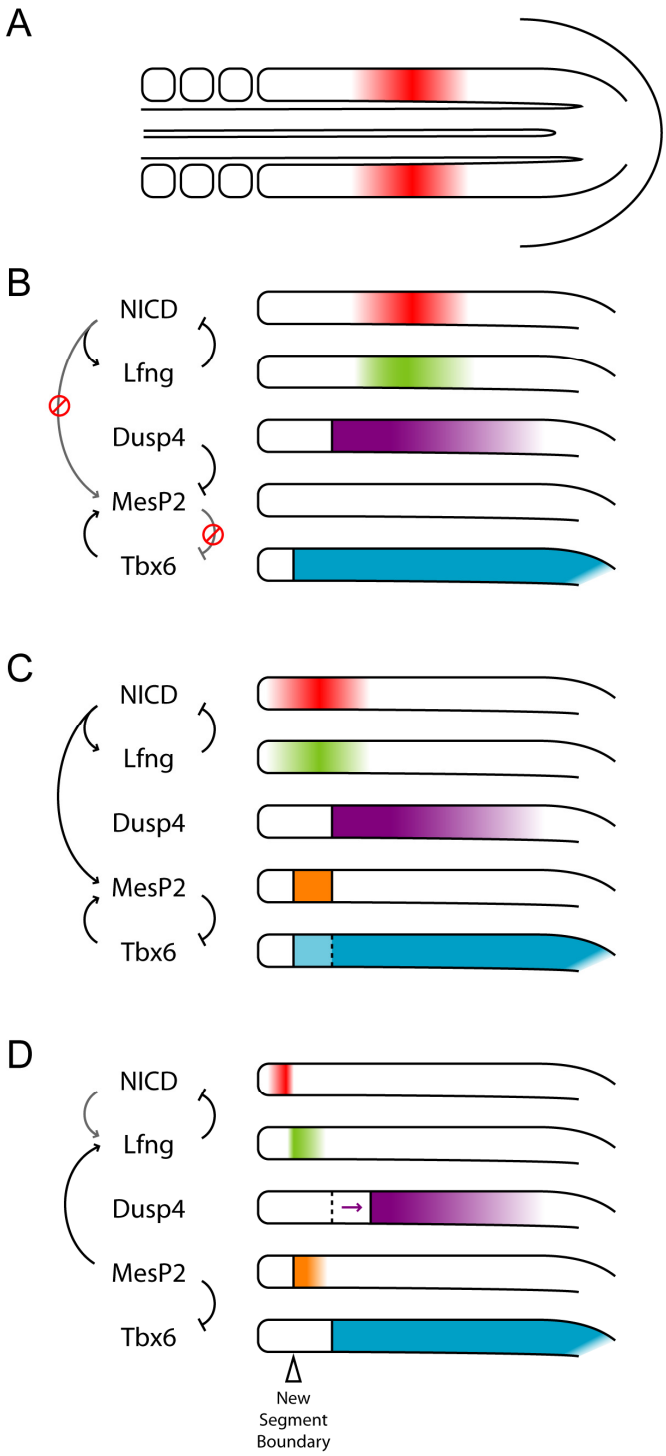


**Figure 1.12: *MesP2* in Segmentation**

Role of *MesP2* in boundary formation. *MesP2* is dependent on *Tbx6* and NICD (Yasuhiko *et al.*, 2008), but is suggested to be restricted by *Dusp4* (Oginuma *et al.*, 2008). Hence, NICD in the posterior PSM does not upregulate *MesP2* (B). When NICD passes over the *Dusp4*-negative, *Tbx6*-positive domain, it upregulates *MesP2* in a domain with sharp boundaries. *MesP2* downregulates *Tbx6* with a sharp anterior

**cont. overleaf**

Interestingly, activated Notch signalling and *Lfng* expression, which is a downstream target of Notch throughout the PSM (Dale *et al.*, 2003) are localised to different AP compartments at this level (Maroto *et al.*, 2005). *Lfng* and *MesP2* become restricted to the anterior compartment of S-1 while NICD is restricted to the posterior compartment of S0, with their interface delineating the segment boundary. This is consistent with a role for *Lfng* as a negative regulator of Notch signalling (Dale *et al.*, 2003). It has been suggested that *MesP2* may drive the suppression of Notch signalling in the anterior compartment by acting on *Lfng*. Indeed, in *Lfng* knockout mice, *MesP2* is expressed in the rostral PSM but does not suppress Notch signalling, while in *MesP2* knockout mice the segregation of *Lfng* and NICD into anterior and posterior compartments is not achieved (Morimoto *et al.*, 2005). A similar effect is seen in the chick PSM, as *Lfng* is restricted to the anterior compartment at S-1 while other notch signalling targets (e.g. *Hairy1*) are restricted to the posterior compartment at S0 (Maroto *et al.*, 2005), however in contrast to the mouse, chick MesP genes are expressed in the posterior

**Figure 1.12: *MesP2* in Segmentation cont.**

boundary (C). *MesP2* activates *Lfng* with a sharp rostral boundary, which suppresses Notch signalling, forming a sharp boundary between the NICD-positive caudal region of S0, and the *MesP2*-positive, *Lfng*-positive rostral region of S-1. The rostral domain of *Dusp4* regresses with the determination front as the axis extends (D). Previous and subsequent waves of NICD and *Lfng* omitted for clarity. (B-D after Oginuma *et al.*, 2008 and Morimoto *et al.*, 2005).

compartment (Buchberger et al, 2002). This discrepancy remains to be explained.

### *Myogenic Regulatory Factor (MRF)*

The third family of bHLH transcription factors relevant to the maturation of the PSM is the myogenic regulatory factor (MRF) subfamily. This group of four closely related proteins – *MyoD* (Davis *et al.*, 1987), *Myf5* (Braun *et al.*, 1989), *MRF4* (Rhodes and Konieczny, 1989) and *Myogenin* (Wright *et al.*, 1989) – have important roles as master regulatory genes for the myogenic lineage (Arnold and Braun, 2000)

The earliest MRF family proteins to be expressed is *Myf5* (Ott *et al.*, 1991). The regulation of *Myf5*, as with other MRF genes, is complicated, although it has been established that *Pax3* is sufficient to induce activation (Maroto *et al.*, 1997, Bajard *et al.*, 2006), however *Myf5* can be activated in the absence of *Pax3*. *Myf5* is also regulated by SHh and Wnt signalling (Borello *et al.*, 2006). In mouse, the earliest expression of *Myf5* is at E8.0 (Ott, 1991). Its expression along the rostrocaudal axis begins in newly formed somites, (Teboul *et al.*, 2002) although some assays suggest that it may be expressed in the PSM (Cossu *et al.*, 1996). In the chick, *Myf5* is expressed throughout the PSM and even in the primitive streak (Kiefer and Hauschka, 2001).

It has been observed that *Myf5* is directly regulated by *Pax3* (Bajard *et al.*, 2006), although *Myf5* is still produced in the absence of *Pax3*. However, in double mutants the somite-derived musculature does not form (Tajbakhsh *et al.*, 1997), suggesting *Pax3* and *Myf5* are may represent parallel, interacting factors required for initiation of the myogenic cascade.

Interestingly, *Myf5* is unusual among the other MRF genes in that it is expressed in cells not associated with the myogenic lineage, in the nervous system (Tajbakhsh and Buckingham, 1995), despite the fact that ectopic *Myf5* is capable of inducing myogenesis in the neural tube (Delfini and Duprez, 2004).

## The T-Box Family

Transcription factors related to the gastrulation factor *Brachyury* (also known as *T*), sharing the T-box DNA binding domain, form the *Tbx* gene family. These genes are involved in the development of a range of tissues, including heart, limbs, pharyngeal arches, and the paraxial mesoderm (Wilson and Conlon, 2002). A number of *Tbx* genes are involved in the development of the paraxial mesoderm, including *Tbx6* and *Tbx18*.

### *Tbx6*

*Tbx6* is expressed in the primitive streak and early paraxial mesoderm. As development procedes it is restricted to the tailbud and presomitic mesoderm, but is excluded from the notochord (Chapman *et al.*, 1996). The precise regulation of *Tbx6* is unclear, but it appears that separate regulatory elements

are associated with its expression in the primitive streak and presomitic mesoderm. In the paraxial mesoderm, *Tbx6* is regulated by Notch signalling via RBP-jκ (White *et al.*, 2005).

*Tbx6* appears to be vital for specification of paraxial mesoderm identity in cells emerging from the tailbud, as loss of *Tbx6* results in the paraxial tissues differentiating into ectopic neural tubes (Chapman and Papaioannou, 1998). A hypermorphic *Tbx6* allele, *rib-vertebrae*, was identified which has helped to elucidate *Tbx6* function (Beckers *et al.*, 2000; Watabe-Rudolph *et al.*, 2002). The *Tbx6* hypomorph exhibits defects in Notch signalling, most significantly a complete impairment of the Notch ligand *Delta1*. Analysis of the *Delta1* promoter revealed that it is directly activated by both *Tbx6* and canonical Wnt signalling (Hofmann *et al.*, 2004, White and Chapman, 2005, 2005), demonstrating that *Tbx6* is both upstream and downstream of Notch signalling. *Tbx6* and activated *Notch* signalling are also required for upregulation of *MesP2* in the rostral PSM (Yasuhiko *et al.*, 2008). Interestingly, while the signal for downregulating *Tbx6* at the mRNA level is not clear, *MesP2* appears to be required for the rapid destruction of *Tbx6* protein via the ubiquitin-proteasome pathway, and in the absence of *MesP2*, *Tbx6* protein and *Delta1* expression are extended rostrally in the paraxial mesoderm (Oginuma *et al.*, 2008). Other targets of *Tbx6* remain to be identified (Wardle and Papaioannou, 2008).

### *Tbx18*

*Tbx18* is expressed in the rostral PSM with a characteristic striped expression, corresponding to the presumptive anterior somite (Kraus *et al.*, 2001, Tanaka and Tickle, 2004). In mouse, expression in the anterior somite is maintained, resulting in a complementary pattern to *Uncx4.1* in the posterior somite, wheras in the chick this expression appears to be lost comparatively early.

In mice, the loss of *Tbx18* does not appear to affect formation of segmental boundaries, as both loss- and gain-of-function mutants form normal epithelial somites. (Bussen *et al.*, 2004). In contrast, in the chick, electroporation and grafting experiments demonstrate that *Tbx18* is capable of driving ectopic boundaries (Tanaka and Tickle, 2004). However, unlike *EphA4*, *Tbx18* is not a target of *Meso1* (Watanabe *et al.*, 2009).

A recent report showed that *Tbx18* physically interacts with *Pax3* to alter transcriptional activity, and that *Pax3/Tbx18* double mutants have an exacerbated skeletal phenotype (Farin *et al.*, 2008). Since *Pax3* is rapidly lost in the sclerotome, this is likely to be an important interaction in the rostral PSM and early somites, and may underlie the effect of *Pax3* on the formation of the axial skeleton (Schubert *et al.*, 2001)

## Snail

The Snail family is a small group of zinc-finger transcription factors associated with epithelial-to-mesenchymal transitions (Barrallo-Gimeno and Nieto, 2005).

*Snail* is required for formation of mesoderm, and mice deficient in *Snail* fail to undergo gastrulation (Carver *et al.*, 2001), however this seems to reflect the role of *Snail* as a mediator of cell behaviour rather than cell identity (Barrallo-Gimeno and Nieto, 2005).

In the chicken embryo, *Snail2* is expressed in the PSM (Sefton *et al.*, 1998) and is dynamically regulated (Dale *et al.*, 2006). Expression is generally highest throughout the caudal PSM (although this appears to oscillate in phase with the segmentation clock), although expression of a rostral band is maintained in the rostral PSM near the presumptive segmental boundary. It has been shown that ectopic *Snail2* can prevent epithelialisation (Dale *et al.*, 2006), consistent with a role in maintaining the mesenchymal identity of the PSM. However, ectopic *Snail2* also appears to interfere with the segmentation clock, raising the possibility that it may interact with signalling in the rostral PSM (Dale *et al.*, 2006, Morales *et al.*, 2007).

## Cell Adhesion and the Extracellular Matrix

As the mechanism by which tissues establish and maintain their structure, cell adhesion has a crucial role in the morphogenesis of all multicellular organisms. Even small differences in concentration of adhesion molecules between cell populations is sufficient to cause profound changes in cell distribution and structure (Foty and Steinberg, 2004), and the establishment of highly organised tissue structures such as epithelia requires a complex process of directed cell adhesion driven by adhesion molecules and dynamic

restructuring of the extracellular matrix (Martins *et al.*, 2009). In the case of the paraxial mesoderm, three cell adhesion systems are particularily important and subject to dynamic regulation throughout somitogenesis: NCAM, N-cadherin, and the extracellular matrix protein fibronectin with its cellular receptors, integrins.

## NCAM

The immunoglobulin superfamily (IgSF) of proteins is a large group of diverse molecules which each contain at least one immunoglobulin (Ig) domain, a protein structure involved in protein-protein and protein-ligand interactions, epitomised by the immunoglobulins (i.e. antibodies). However, the Ig domain has been put to a wide variety of uses, from foreign antigen recognition in the immune system to ligand binding in cell signalling receptors, and even intracellular proteins like the muscle kinase titin (Barclay, 2003). The IgSF also includes a family of Ig domain containing proteins involved in cell adhesion (IgCAMs), the best characterised of which is Neural Cell Adhesion Molecule (NCAM).

### *Adhesion, Expression, and Structure*

The adhesive role of NCAM is well characterised. NCAM can drive aggregation in non-aggregating cell lines (Mege *et al.*, 1988), and can even promote aggregation in cell-free assays using lipid vesicles (Hoffman and Edelman, 1983). As its name suggests, NCAM appears to be predominantly involved in cell-cell interactions in the nervous system. However, NCAM is expressed in all germ layers throughout development, including the

developing intestine (Probstmeier *et al.*, 1990), skin and feather rudiments in chicken (Marsh and Gallin, 1992), the developing kidney (Markovic-Lipkovski *et al.*, 2007) and both cardiac and skeletal muscle (Watanabe *et al.*, 1992; Reyes *et al.*, 1991). In the paraxial mesoderm, NCAM is a marker of PSM maturation (Duband *et al.*, 1987) and is expressed throughout the dorsoventral axis of the PSM and early somites, including presumptive sclerotome (Lyons *et al.*, 1992). As differentiation of somites continues, NCAM is restricted to the myogenic lineage and various isoforms of NCAM are sequentially and locally activated, potentially reflecting a varying role for myoblast migration (Lyons *et al.*, 1992), myotubule fusion (Suzuki *et al.*, 2003), and formation of neuromuscular junctions (Rafuse *et al.*, 2000). In mature muscle, NCAM is only expressed at the neuromuscular synapses. It is not expressed on either mature muscle or satellite cells, but is transiently activated during differentiation of myoblasts from satellite cells, and has been suggested as a marker of muscle regeneration (Dubois *et al.*, 1994).

In addition to it's role in cell-cell adhesion, NCAM has also be described as important in cell-matrix adhesion (Cole *et al.*, 1986), via interactions with heparan sulphate proteoglycans (HSPGs; fig. 1.13b).

Studies in the rat suggest that there may be as many as 27 distinct isoforms of NCAM produced by alternative splicing of the mRNA transcript at the exon7-exon8 junction, the exon 11-exon 12 junction, and the 3' terminus (Reyes *et al.*, 1991). Specific isoforms appear to be associated with particular

functions, for example the inclusion of a muscle-specific domain correlates with myotube fusion (Yoshimi *et al.*, 1993). However the most relevant variations concern the 3' intracellular/transmembrane domain, giving rise to three major isoforms. The full transcript produces a ~180 kDa protein (NCAM-180) which contains a large cytoplasmic domain (Murray *et al.*, 1986). This isoform is not detected in early morphogenesis, and is predominantly restricted to the nervous system, specifically associated with neurite outgrowth. The cytoplasmic domain is subject to phosphorylation, and is thought to be important for the adhesion-dependent activation of the NF-kB transcription factor (Little *et al.*, 2001). A shorter isoform, resulting from the deletion of exon 18, produces a ~140kDa protein lacking the cytoplasmic tail (NCAM-140). This isoform is expressed throughout embryonic development, associated with several tissues including the nervous system and the musculature. Despite lacking intracellular phosphorylation domains, NCAM-140 may still take part in cell signalling by associating with FGF receptors (Williams *et al.*, 1995, Carafoli *et al.*, 2008; fig 1.13a). The cytoplasmic domains of both NCAM-180 and NCAM-140 interact with microtubules, although NCAM-180 interacts with a greater range of cytoskeletal proteins (Buttner *et al.*, 2003; fig. 1.13a). The third major isoform lacks the cytoplasmic and transmembrane domains, produces a ~120kDa protein, and is linked to the cell membrane via phosphotidylinositol (Hemperly *et al.*, 1986). Its function is associated almost exclusively with the foetal and mature brain, appearing in chickens around day 9, and continuing into adulthood.

A second IgCAM which forms a small subfamily with NCAM has been identified (Yoshihara *et al.*, 1997). NCAM2 has a very similar structure to NCAM, exists in both a transmembrane and GPI-linked isoforms, and is thought to have similar binding properties. In contrast to NCAM, it is not subject to polysialylation (Cremer *et al.*, 1994). To date, it's only known role is in differential fasciculation of the olfactory nerve, and while it is also detected in the developing retina and brain, it is not expressed in any non-neural tissues throughout development (Yoshihara *et al.*, 1997).

Strangely, despite its broad expression pattern NCAM appears to be more or less dispensible for correct body patterning, with *NCAM*-null mice being viable

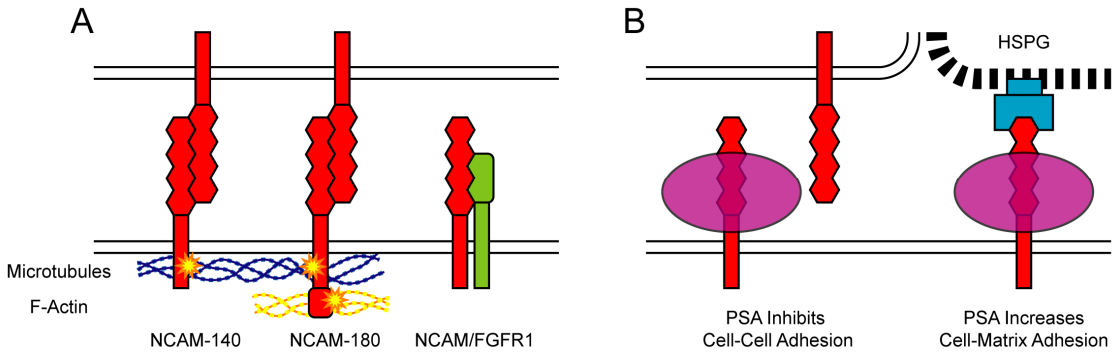


**Figure 1.13: Structure and Function of NCAM**

A: NCAM is a transmembrane Ig-like cell adhesion molecule (red). It mediates cell-cell adhesion via homophilic binding. NCAM is connected to the microtubule network (blue), and the 180kD isoform also binds to the actin cytoskeleton (yellow; Buttner *et al.*, 2003). Cis interactions have been described between cell surface molecules such as FGFR1 (green, Williams *et al.*, 1995). B: NCAM is subject to posttranslational modification with polysialic acid (PSA, pink). This large carbohydrate chain is thought to interfere with homophilic binding and disrupt cell-cell adhesion. Additionally, it may also promote cell-matrix adhesion by allowing NCAM to bind to ECM molecules such as HSPGs (blue, Storms and Rutishauser, 1998).

with only a subtle spatial learning phenotype (Cremer *et al.*, 1994). Muscular innervation and regeneration have been studied in detail in the *NCAM*-null background (Moscoso *et al.*, 1998; Rafuse *et al.*, 2000) which uncovered only minor perturbations of both. It is possible that the mild phenotype is due to functional redundancy with other adhesion molecules. Interestingly, transgenic mice producing a mutant form of *NCAM* lacking the cytoplasmic and transmembrane domain, producing a secreted form of the protein, exhibited a severe, dominant, embryonic lethal phenotype with severely disrupted segmentation (Rabinowitz *et al.*, 1996). The same phenotype was observed against an *NCAM*-null background, strongly suggesting that heterophilic binding partners for NCAM play an important role in this phenotype. However, whether this phenotype is a result of interference with cell adhesion or effects on other binding partners (such as HSPG or the FGF receptor) has not been investigated.

## *Glycosylation*

Sialic acids are a family of sugar residues derived from neuraminic acid (Blix *et al.*, 1957). In most cases sialic acids represent the terminal residue on sugar chains (Schauer, 2000). Using nomenclature based on the carbon number of each sugar residue, sialic acids are usually connected by α2-3 or α2-6 linkage, however homopolymers of sialic acid based on α2-8 linkage do occur. This unique and extensive carbohydrate modification is known as polysialic acid (PSA) (Finne, 1982). PSA assembly is dependent on two sialyltransferase enzymes, *ST8SiaII* and *ST8SiaIV* (Eckhardt *et al.*, 1995;

Scheidegger *et al.*, 1995). The most abundant acceptor for PSA in mammals appears to be NCAM, as mice deficient in NCAM show a near complete absence of α2-8 linked sialic acid (Cremer *et al.*, 1994), and it is certainly the most studied example of PSA. However, six other acceptors for PSA have been identified; including the scavenger receptor *CD36* (although only when secreted during lactation, Yabe *et al.*, 2003), the co-receptor *Neuropilin-2* (Curreli *et al.*, 2007), the α-subunit of the voltage gated sodium channel (Zuber *et al.*, 1992), and the two sialyltransferases involved in constructing PSA (Close and Colley, 1998). Interestingly, a second Ig-like cell adhesion molecule involved in neurogenesis, SynCAM, has also recently been described as polysialylated (Galuska *et al.*, 2010).

The physical and electrochemical properties of PSA make it a good candidate for a negative regulator of cell adhesion (reviewed in Rutishauser *et al.*, 1988). The site of glycosylation is proximal to the membrane, and is thought to interfere with homophilic binding to neighbouring cells (fig. 1.13b). However, polysialylation also appears to modulate the adhesion of NCAM to extracelullar matrix molecules such as HSPGs (Storms and Rutishauser, 1998), suggesting that polysialylation may represent a shift from cell-cell adhesion toward cell-matrix adhesion. However, classical studies of PSA-NCAM show that they may are involved in fasciculation of nerve cells (Rutishauser *et al.*, 1988) as well as maintaining cell-cell contacts during neurogenic chain-migration (Hu, 2000), suggesting that the relationship between PSA-NCAM and cell-cell adhesion may not be straightforward. It is

also possible that polysialylation of NCAM may affect cis-interactions, such as formation of ternary complexes with other adhesion molecules (Kadmon *et al.*, 1990) and disruption of signalling (Williams *et al.*, 1995).

The polysialylation of NCAM is subject to developmental regulation (Ong *et al.*, 1998), particularily in relation to neurogenesis (Boisseau *et al.*, 1991). It's abundance seems to peak during late embryogenesis, consistent with a role in axon guidance and synapse formation, however it has also been implicated in the migratory activity of neuroblasts during adult neurogenesis (Bonfanti, 2006). It's role in non-neurogenic tissues has not been explored, although a polysialylated form of NCAM (known as CD56) has been detected on NK cells in the immune system (Warren *et al.*, 1993), and as a marker of myogenesis in muscle satellite cell cultures (Bosnakovski *et al.*, 2008). In both adult myogenesis and neurogenesis, transient expression of PSA-NCAM seems to be transiently upregulated by cells undergoing differentiation (Bosnakovski *et al.*, 2008; Gascon *et al.*, 2008).

Sialic acid is of crucial importance in development, with knockouts for an enzyme required for its synthesis lethal at around E8.5 (Schwarzkopf *et al.*, 2002). Double knockouts of the two polysialyltransferase enzymes, representing a PSA knockout, exhibit severe defects in brain patterning and show postnatal lethality (Weinhold *et al.*, 2005). Intriguingly, while the sialyltransferase double-knockout is more severe than the *NCAM*-null, the triple-knockout closely resembles the *NCAM*-null phenotype. This suggests

that the PSA-null phenotype is caused by inappropriate activity of NCAM, further supporting the idea that polysialic acid is a negative modulator of NCAM activity (Weinhold *et al.*, 2005).

### *Regulation*

Cell adhesion is vitally important in establishing both the structure and functionality of the vertebrate nervous system. For this reason, genetic regulation of cell adhesion molecules represents an important mechanism for translating positional identity conferred by transcription factors into functionally distinct tissues (Edelman and Jones, 1998). The NCAM promoter has been studied in detail using ChIP, in vitro and transgenic studies, and has been identified as a direct target for various Hox (Jones *et al.*, 1992) and Pax (Chalepakis *et al.*, 1994, Holst *et al.*, 1997) transcription factors. Analysis of the *Pax3* mutant *splotch* has also highlighted altered NCAM expression (Bennett *et al.*, 1998). Interestingly, an upregulation of NCAM in the neural tube has been reported, with a differing isoform profile (Moase and Trasler, 1991), but this may be attributable to the fact that the mutant *Pax3* protein binds to the *NCAM* promoter *in vitro* while the wild type *Pax3* does not (Chalepakis *et al.*, 1994).

The expression pattern of NCAM during segmentation and early specification of the myotome (Lyons *et al.*, 1992) closely follows the expression pattern of *Pax3* (Schubert *et al.*, 2001). Interestingly, it was recently shown that ectopic *Pax2* is capable of promoting NCAM expression, and is required for

upregulation of NCAM in response to ectopic *Meso1* (Watanabe *et al.*, 2007). Together, these point to the idea that Pax genes may be important regulators of NCAM in the paraxial mesoderm.

Pax genes have also been discussed in the control of polysialylation of NCAM. Initial reports showed precocious polysialylation of NCAM was observed in *splotch* mutants (Neale and Trasler, 1994). However, *Pax3* has been shown to be a direct regulator of *ST8SiaII*, and can drive polysialylation in cell culture (Mayanil *et al.*, 2001). Furthermore, a later examination of the *splotch2H* mutant revealed a downregulation of PSA-NCAM (Glogarova and Buckiova, 2004). These varying and often contradictory reports may reflect a complex relationship between *Pax3* and PSA-NCAM in different tissues, and at different time points throughout development.

## N-Cadherin

Cadherins are a family of calcium-dependent cell adhesion molecules which have important roles in cell adhesion and cell migration. They are important components of adherens junctions, localised adhesion complexes which mechanically connect the actin cytoskeleton of neighbouring epithelial cells (Takeichi, 1988). A specialised form of adherens junction called the zonula adherens is particularily associated with epithelia, forming an apical boundary.

As its name suggests, epithelial cadherin (E-cadherin) is classically associated with the adherens junctions of epithelia. In contrast, N-cadherin is

often considered a marker of mesenchymal cell identity. The switch from E- to N-cadherin is often taken as a marker of epithelial-to-mesenchymal transition (De Wever *et al.*, 2008). However, N-cadherin may also be associated with epithelial cells at certain points in development, although usually in addition to E-cadherin. Epithelia in which the zonula adherens is mediated primarily by N-cadherin are extremely unusual, but there are two famous examples: the neuroepithelium of the neural tube, in which the switch from E-cadherin to N-cadherin is required for neurulation but does not cause EMT; and the somites, which form epithelial structures without the involvement of E-cadherin.

N-Cadherin is specifically associated with the mesoderm (Hatta and Takeichi, 1986). Like NCAM, the molecule is present throughout the PSM, but is markedly upregulated at the determination front (Duband *et al.*, 1987). However, N-cadherin only assumes a polarised distribution characteristic of adherens junctions at around S-1 (Martins *et al.*, 2009). As segmentation occurs and cells are recruited to the somitic epithelium, N-cadherin and F-actin are concentrated at the internal (apical) surface of cells (Duband *et al.*, 1987, Martins *et al.*, 2009, Nakaya *et al.*, 2004). α- and β-catenins are also localised to this region, suggesting the establishment of functional adherens junctions (Linask *et al.*, 1998).

Using both knockout mice and function-blocking antibodies in chick embryos, it was found that loss of N-cadherin function resulted the formation of

supernumary somites, orientated mediolaterally in chick and dorsoventrally in mouse (Linask *et al.*, 1998, Radice *et al.*, 1997). Interestingly, the somites had correctly localised β-catenin and an appropriate structure, suggesting that some other adhesion molecule was replacing N-cadherin in somitogenesis.

A second cadherin has been described in mouse somitogenesis, Cadherin-11. It is upregulated in the rostral PSM throughout the DV axis. In contrast to N-cadherin, it is localised to the ventral somite, and is transiently maintained as the sclerotome loses its epithelial character, before being downregulated (Kimura *et al.*, 1995). Knocking out both N-cadherin and Cadherin-11 resulted in the formation of multiple small clusters without epithelial morphology (Horikawa *et al.*, 1999). Interestingly, the cells exhibited correct *Uncx4.1* staining, indicating that epithelialisation of the somite is not required for acquisition of correct AP patterning.

Because of its role in morphogenesis, *Paraxis* has been suggested to regulate N-cadherin, although no direct connection between *Paraxis* and N-cadherin has been established. However, the ability of *Paraxis*-transfected cells to incorporate into the somite epithelium has been shown to depend on Rho-family small GTPases, which may regulate the formation of adherens junctions (Nakaya *et al.*, 2004).

## The Differential Adhesion Hypothesis

Cell adhesion underpins vertebrate morphogenesis. It is integral to the way in which tissues and organs are defined and distinguished from one another. The manner in which tissues differentiate from each other during development was first examined by comparing the ability of embryonic tissue fragments to recombine (Holtfreter, 1939) and even reconstitute themselves after dissociation (Townes and Holtfreter, 1955). There have been several mechanisms proposed by which cells from dissimilar tissues might segregate. Most suggest that different tissues express different homophilic adhesion molecules, such as different cadherins (Takeichi, 1990), and segregation is driven by adhesion molecule subtype specificity.

An alternative hypothesis suggests that different cell surface concentrations of the same adhesion molecules are sufficient to drive segregation and reconstitution of cell populations in a manner analogous to the dispersion of an emulsion of two immiscible liquids (Steinberg, 1970). This model is called the Differential Adhesion Hypothesis (DAH), and has been shown to accurately predict the behaviour of tissue explants in recombination assays (Foty and Steinberg, 2005). The apparent power of DAH in modelling cell behaviours in tissues underscores the necessity to consider not only the relative distributions of different cell adhesion molecules during morphogenesis, but their relative abundance on the cell surface.

## Integrins and Fibronectin

The extracellular environment has an important role in morphogenesis. Intercellular spaces are largely filled with networks of proteins and polysaccharides, collectively termed the extracellular matrix (ECM), which has a wide range of functions both in mature tissues and throughout development. The ECM has a range of functions in morphogenesis; providing structural support and a substrate for migration or cellular shape change, delineating the boundaries between different tissues and allowing tissue-scale shape changes to occur, and even participating in cellular signalling and cell differentiation.

While there are many cell surface proteins involved in cell-matrix interactions, one of the most important and best characterised is the Integrin family, which link the ECM into the intracellular cytoskeleton both structurally and as a signalling transducer. Similarily, while there are a wide range of extracellular matrix components, one of the best studied and arguably one of the most developmentally relevant is fibronectin, which is also thought to be the most important for cell-matrix interactions. The relationship between integrins and fibronectin are complex and dynamic, and play integral roles in development.

### *Integrins*

The Integrins are a family of heterodimeric cell surface receptors, named for their function in integrating the molecules of the extracellular matrix with the intracellular cytoskeleton (Hynes, 1987). Each heterodimer contains one of

18 possible α- and one of eight possible β-subunits (Hynes, 2002). This variety of receptor structures is further expanded by a range of possible alternative splicing and post-translational modification events.

The classic ligands for integrins are extracellular matrix components, including collagens, fibronectin, and laminins among many others. However, some integrins may also bind to other cell surface "counter-receptors", including cell adhesion molecules such as E-cadherin or V-CAM (Plow *et al.*, 2000). Almost all cell types express some combination of integrins, and their functions are implicated in a wide variety of biological processes throughout development and adulthood.

Broadly speaking, and considering only cell-matrix interactions, there are two main families of integrin receptor: those that contain the β1 subunit, and those that contain the αv subunit. However, mouse knockouts of αv have a comparatively mild phenotype (Bader *et al.*, 1998), while mice lacking the β1 subunit failed to form a compacted inner cell mass and died shortly after implantation at E5.5 (Stephens *et al.*, 1995). While heterodimers from both families recognise ligands such as fibronectin, there are also family-specific binding profiles; for example, laminin and collagen are primarily recognised by β1-containing heterodimers, while vitronectin and fibrinogen by αv containing heterodimers. It is also known that heterodimer combinations may play distinct roles in terms of adhesion and signal transduction (Hynes, 2002).

## *Fibronectin*

Fibronectin is a large extracellular matrix glycoprotein which plays an important role in dynamic interactions between cells and the matrix (Hynes, 2009). It is highly expressed in a wide variety of tissues throughout development, but in adulthood it is primarily associated with wound healing and haemostasis (Vartio *et al.*, 1987). It is synthesised as a single gene with multiple exons, and is alternatively spliced to produce several isoforms. Fibronectin is secreted as a dimer, each subunit containing binding sites for the extracellular matrix components heparin and collagen, and at least fifteen Type 3 Fibronectin repeats, which form the binding domains for their cellular receptors, the Integrins. These soluble units are assembled into fibrils in the pericellular space (McDonald, 1988). Fibronectin has been associated with the basal lamina, and in many tissues forms an organised sheet at tissue boundaries, however a fibronectin matrix may be formed in the absence of definitive laminin-containing basal laminae (Thorsteinsdottir, 1992).

### Fibronectin Assembly

Unlike other ECM components, such as collagen, which will readily assemble into polymers, fibronectin does not spontaneously assemble (McDonald, 1988), but rather depends on interactions with adjacent cells (fig. 1.14a). This process is mediated by Integrins (Wu *et al.*, 1993). Interestingly, while a wide range of Integrins are known to function as receptors for fibronectin, only heterodimers containing α5 and αv have been shown *in vitro* to have

fibrillogenic activity (Yang and Hynes, 1996), and mice deficient in both α5 and αv exhibit severe deficiencies in the fibronectin matrix (Yang *et al.*, 1999).

The ability of integrins to bind fibronectin, and therefore assemble the fibronectin into a matrix, is subject to allosteric regulation (Mould, 1996). Binding of intracellular factors such as talin produce transmembrane conformational changes which allow integrins to bind their ligands in the extracellular matrix. Hence, fibronectin assembly can be regulated dynamically by intracellular signalling events.

Intriguingly, fibrillogenesis is also related to the actin cytoskeleton. It has been known for some time that fibronectin fibrils tend to form in the same direction as actin stress fibres (Hynes and Destree, 1978), and disruption of the actin cytoskeleton with cytochalaisin B inhibits fibrillogenesis (Ali and Hynes, 1977). It has also been shown that tension applied to fibronectin subunits by the actin cytoskeleton reveals cryptic binding sites that facillitate fibrillogenesis (Zhong *et al.*, 1998). Knockout studies have shown that there is a degree of redundancy between integrin isoforms in this process, as no single knockout exhibits marked loss of fibronectin. However, double knockout analyses show that integrin α5 and αv double-knockout mice exhibit a profound loss of fibronectin in the early embryo (Yang *et al.*, 1999).

In single-strain cell culture assays, a single cell type is evidently sufficient both to produce and assemble the fibronectin matrix. However, *in vivo*, where multiple cell types exist together, it is not absolutely necessary that soluble

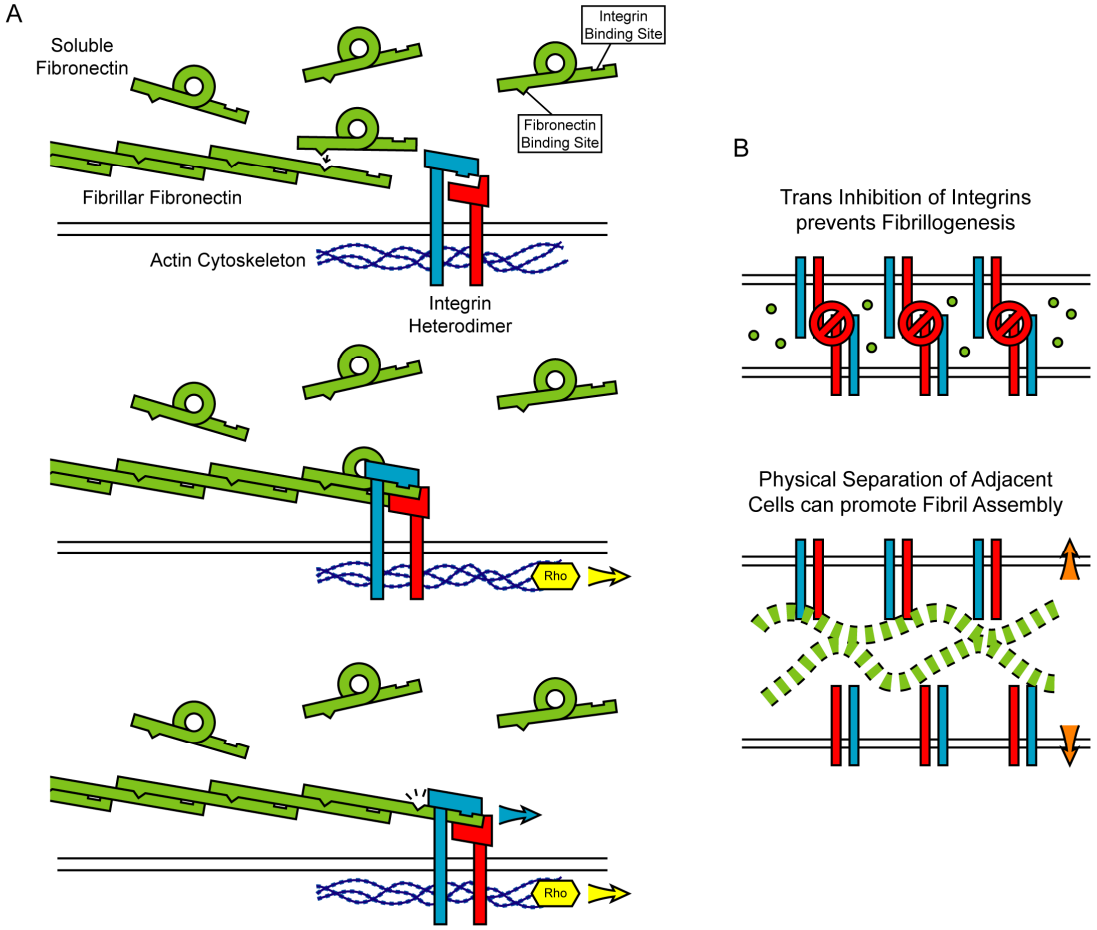


**Figure 1.14: The Role of Integrins in Fibronectin Assembly**

A: Fibronectin assembly is dependant on tension applied to fibronectin monomers by the actin cytoskeleton (blue) via integrin receptors (red and blue). Soluble fibronectin (green) contains a fibronectin binding site and an integrin binding site. When tension is applied to the fibronectin monomer, it reveals a cryptic fibronectin binding site, allowing extension of the fibrillar fibronectin polymer. This may be driven by Rho GTPase-activated cytoskeletal changes mediated by integrins (yellow, Zhong *et al.*, 1998). B: The ability of integrins to assemble fibrils may limited by trans-inhibition by integrins on neighbouring cells (Julich *et al.*, 2009).

fibronectin must be assembled by the same cells which produce it. In the case of the chick embryo, it apppears that surface ectoderm represents a much more abundant source of fibronectin than the paraxial mesoderm (Rifes *et al.*, 2007). Furthermore, PSM explants cultured with surface ectoderm or exogenous soluble fibronectin recover much more quickly from proteolytic degradation of the fibronectin matrix.

### *Fibronectin and Integrins in Embryogenesis*

Studies in *Xenopus* (Davidson *et al.*, 2006) have shown that fibronectin is dynamically regulated in the early embryo. The earliest known function of fibronectin in embryogenesis is the morphogenesis of the mesoderm during gastrulation. In the chick embryo, blockade of fibronectin using antibodies prevents migration of mesoderm, causing it to accumulate under the primitive streak (Harrisson *et al.*, 1993), and preventing the formation of the paraxial mesoderm. Studies in *Xenopus* reveal dynamic remodelling of the fibronectin matrix during gastrulation (Davidson *et al.*, 2006), and it's disruption causes a complete failure of mesodermal migration (Thiery *et al.*, 1985). In fibronectin knockout mice, gastrulation and mediolateral mesodermal migration occurs normally, however subsequent axial and paraxial morphogenesis was disturbed (George *et al.*, 1993). Specifically, while markers of paraxial mesoderm and somite derivatives were detected in appropriate domains, correctly epithelialised somites were not observed (Georges-Labouesse *et al.*, 1996). This is consistent with observations in the chick, where accumulation of fibronectin network is associated with morphological maturation of the PSM

and formation of an epithelial somite (Duband *et al.*, 1987), and that morphological somite formation is impaired when the PSM fibronectin matrix is abolished by enzymatic digestion or antibody interference (Rifes *et al.*, 2007).

A coherent fibronectin matrix is restricted to the surface of the PSM (Julich *et al.*, 2009), with only short strands of fibronectin present within the PSM itself (Duband *et al.*, 1987, Davidson *et al.*, 2006). Interestingly, while a full basal lamina is eventually assembled around the somite, laminin expression is patchy and incomplete at the PSM boundary and around the first formed somite (Duband *et al.*, 1987, Martins *et al.*, 2009). When boundary formation occurs, a fibronectin matrix is rapidly assembled within the boundary. A recent study in zebrafish suggested that homotypic trans-interactions between integrins on neighbouring cells prevent fibronectin assembly (Julich *et al.*, 2009; fig 1.14b). They showed that signalling through *EphA4*, which occurs naturally at the anterior boundary of newly forming somites, is sufficient to drive fibrillogenesis, even in tissue which does not normally produce boundaries. However, they also noted that merely performing tissue grafts between embryos was enough to drive fibronectin matrix assembly in a quarter of cases; physically separating cells may be sufficient to deactivate this inhibition of integrin activity. However, grafting experiments are a staple of chick embryology, and have been used repeatedly within the PSM (Aoyama and Asamoto, 1988, Dubrulle *et al.*, 2001) without induction of ectopic borders (Sato *et al.*, 2002), and while acute effects on fibronectin have not been

investigated it does not seem likely to represent a mechanism for boundary maintenance.

Interestingly, Rho family GTPases have been shown to promote fibrillogenesis by exerting tension on the actin cytoskeleton (Zhong *et al.*, 1998; fig. 1.14a). Given the role of Rho family GTPases in formation of an epithelium (Nakaya *et al.*, 2004), and the involvement of eph-ephrin signalling in their regulation (Watanabe *et al.*, 2009), this represents another potential connection between segmentation and fibrillogenesis.

# Materials and Methods

## Embryo Techniques

Fertilised White Leghorn hens eggs were provided by Winter Farm and Henry Stewart in England. Upon arrival, eggs were stored at 16ºC before incubation at 38.5ºC in a humidified incubator. Embryos were staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951), although we have consistently found our eggs developed slightly slower than they recorded (Tenin *et al.*, 2010). Embryos were dissected and staged in room temperature phosphate buffered saline (PBS), before being treated as described elsewhere.

### Half Embryo Culture

Embryos were cultured *in ovo* to Hamburger-Hamilton stage 11 (HH11), and then collected at room temperature and transferred to Leibovitz L15 (Invitrogen) on ice. Embryos were pinned out with entomology pins and bisected down the midline with electrolytically sharpened tungsten wire. Embryo halves were transferred to Isopore 1.2µm RTTP filters (Millipore) and randomly assigned to each experimental condition. Filters were then floated on OptiMEM (Invitrogen) with 5% foetal bovine serum (BioSera), supplemented with L-glutamine (Invitrogen) and gentamicin (Invitrogen). For drug treatments, one half was treated with a drug and the other half with a vehicle control. For fix-and-culture experiments, one half was fixed after 15

minutes of culture, and the other half after a defined time period. Explants were fixed in 4% formaldehyde (Sigma) in PBS and processed as normal.

## *In Ovo* Electroporation

DNA was prepared in a 4-10 µg/µl solution with fast green (Sigma). Tetracycline-inducible plasmids (pBI) were co-transfected with a tetracycline-activated transcription factor (reverse-tetracycline transactivator, rtTA) at a ratio of 2:1. Glass micropipettes (Harvard Apparatus) were made from borosilicate capillary tubes using a Flaming/Brown P87 pipette puller. The tip was broken with a set of forceps to allow free flow of DNA solution. A set of platinum electrodes were created with a fixed separation of approximately 10mm. The lower electrode was sharpened in order to pierce the vitelline membrane.

Eggs were cultured to HH6-8, and windowed to allow access to the embryo. Using a glass micropipette, DNA solution was injected between the vitelline membrane and the embryo in the region of the primitive streak. A few drops of Hanks Buffered Saline Solution (HBSS, Invitrogen) or PBS was added to aid conductivity. The electrodes were positioned above and below the primitive streak. Electric pulses were applied across the primitive streak using a BTX ECM830 electroporator. Typical electrical conditions were five 50ms pulses at 10-20v, 500ms apart. 500µl of HBSS or PBS was added to the egg, which was sealed with Parafilm (Pechiney) and returned to a humidified incubator. After culture, embryos were collected as normal.

### *Doxicycline Injection*

Embryos electroporated with inducible constructs were injected with the tetracycline analogue doxicycline (Sigma) 18 hours after transfection. 500µl of HBSS containing 0.1µg/ml doxicycline was injected into the yolk beneath the embryo using a 25G syringe needle (BD). Eggs were resealed and returned to a humidified incubator.

### *Pre-Staining Images*

Electroporated embryos intended for further staining or cryosectioning were photographed to record the pattern of transfection. After fixation, embryos were transferred to PBS with 0.1% Tween20 (PBST, Sigma) and photographed on a Leica MZ-16F and Hamamatsu C4742-95.

## EC Culture

EC culture was performed as described previously (Chapman *et al.*, 2001). Agar-albumen culture dishes were prepared by mixing 0.6% Bacto Agar (BD) in simple saline (123mM NaCl) with an equal volume of thin albumen. For drug treatments, the agar-albumen was allowed to cool slightly before addition of the drug.

Embryos were excised from the yolk on the vitelline membrane supported by rings of filter paper. Care was taken to minimise the yolk attached to the embryo. If necessary, excess yolk was removed with simple saline or HBSS, but this was avoided in embryos under HH8. Embryos were transferred to plates and cultured in a humidified incubator at 38.5ºC, 5% $CO_2$.

### Drug Treatments

Notch pathway signalling was inhibited using the γ-secretase inhibitor LY411575 (synthesised in house). The drug was dissolved in DMSO at 1.5μM, and diluted to a working concentration of 150nM. A 1:10,000 dilution of DMSO was used as a control. Wnt signalling was inhibited using the casein kinase inhibitor CKI-7 (US Biological) dissolved in ethanol at 50mM, and diluted to a working concentration of 200μM. A 1:250 dilution of ethanol was used as a control.

## *In Situ* Hybridisation

### Preparation of antisense RNA probes

Fragments of target genes were amplified by PCR using specific primers designed using sequences from the Ensembl genome browser (http://www.ensembl.org). Usually, fragments were designed to be around 800 base pairs in length, although in some cases (such as *NCAM1*) longer probes were used. Fragments were separated on a 2% agarose gel, extracted and purified using a Qiagen kit. This fragment was then cloned into the TOPO-II (Invitrogen), transformed into Stratagene XL-1 Blue competent cells. Clones were analysed by restriction digest and sequencing to confirm the presence, identity, and orientation of an insert. Appropriate restriction sites and RNA polymerases were selected to produce antisense RNA probes. An *in vitro* transcription reaction was performed using digoxigenin-labelled ribonucleotides.

### Hybridisation

Embryos and explants were fixed in 4% formaldehyde, washed in PBS with 0.1% Tween20 (PBST), and staged into 100% Ethanol for storage at -20ºC. After rehydration with PBST, embryos were subjected to proteinase K digestion and post-fixed with 4% formaldehyde and 0.1% glutaraldehyde in PBST. Embryos were then staged into hybridisation mix (50% formamide, 1.3x SSC, 5mM EDTA, 50µg/ml tRNA, 0.2% Tween20, 0.1% SDS, 100µg/ml Heparin) and incubated at 65ºC for up to 48 hours. At this point, hybridisation mix with 30µl/ml antisense RNA probe was added and allowed to hybridise overnight. The probe was then recovered, and samples thoroughly washed in hybridisation mix and staged into TBS with 0.1% Tween20 (TBST).

Samples were blocked with 2% blocking reagent (Roche) and 20% goat serum (Invitrogen) in TBST, and an alkaline phosphatase conjugated anti-digoxigenin antibody (Roche) was used to detect the RNA probe. After being thoroughly washed with TBST, antibody staining was detected using NBT/BCIP (Promega). Embryos were post-fixed in 4% formaldehyde in PBST, and photographed with a Leica MZ-16 microscope and Jenoptik ProgRes C14 camera.

## Histology

### Sectioning

Samples were equilibrated in 30% sucrose in PBS and mounted in 1.5% LB Agar (Invitrogen) dissolved with 5% sucrose in PBS. Blocks were

cryopreserved in 30% sucrose and flash frozen with dry ice before being stored at -80º for at least 18 hours. Blocks were sectioned at 17µm in a Leica CM3050-S cryostat, and stored at -20ºC until use.

## Immunohistology

Sections were blocked with 10% goat serum in PBS with 0.1% Triton-X (VWR, PBSX) for at least an hour at room temperature. Antibodies were added at appropriate concetrations in 10% goat serum in PBSX, and incubated for four hours at room temperature or overnight at 4ºC. Slides were then washed in PBSX before an appropriate fluorescent secondary antibody (Invitrogen) was applied at 1:250 in 10% goat serum in PBSX. Slides were washed in PBSX and counterstained DAPI before being mounted in hydromount (National Diagnostics) supplemented with 2% DABCO (Sigma) as an anti-fading agent. Sections were imaged with a Leica DM 5000B microscope using a Q-Imaging Retiga 2000R camera.

For Integrin α5, antigen retrieval was performed before the blocking step. Slides were placed in unmasking solution (Vector Laboratories) at 100ºC for 5 minutes, and then treated as normal. For Pax3, 1% milk (Marvel) was substituted for goat serum as a blocking reagent for the primary antibody steps.

## Counterstaining

Slides stained for F-actin using phalloidin conjugated to an appropriate fluorophore (Invitrogen). Phalloidin was added at 1:500 in 10% goat serum in

PBSX along with secondary antibody or alone, and then washed as usual. Slides stained with DAPI were incubated in 1% DAPI in PBS for 30 seconds, and washed with PBSX.

### Antibodies

Monoclonal primary antibodies for Pax3 (Pax3), Pax7 (P3U1), Integrin α5 (A21F7) and PSA-NCAM (5A5) were obtained from the Developmental Studies Hybridoma Bank (DSHB). Mouse monoclonal anitbodies for N-cadherin (Invitrogen), and GFP (Roche) were also used. Rat monoclonal antibodies for Tubulin were a kind gift from Inke Nathke. Rabbit polyclonal antibodies for NCAM and Fibronectin were a kind gift from Sylvie Dufour.

## Molecular Biology

### cDNA Preparation

Total RNA was collected from samples using the RNEasy Mini Kit (Qiagen) for multiple embryo samples, or the RNEasy Micro Kit (Qiagen) for single embryos or explants. RNA was annealed with poly-dT and heaxanucleotide primers, and reverse-transcribed using M-MLV reverse transcriptase (Promega). cDNAs were then purified using the QIAquick PCR purification kit (Qiagen) and eluted in an appropriate volume.

### qRT-PCR

Specific primers were designed using sequences from Ensembl and predicted properties assessed using OligoCalc (http://www.basic.northwestern.edu/biotools/oligocalc.html), and synthesised by Thermo Scientific. Primers were

chosen which had a predicted $T_m$ of 52ºC (Wallace *et al.*, 1979), avoiding self-complimentarity (Serra *et al.*, 1993 and Vallone *et al.*, 1999) and produced a predicted fragment of 250bp. To exclude contamination of the cDNA with genomic DNA, wherever possible one primer was situated on an intron-exon boundary. Primers were validated by using a titration of known concentrations of template DNA, and a melting curve analysis was performed to exclude primer self-dimerisation. qRT-PCR was performed in triplicate using [master mix] and an Eppendorf Realplex$^2$ thermal cycler. *GAPDH* was used to control for sample concentration.

## Molecular Cloning

Specific primers were designed using sequences from Ensembl and predicted properties assessed using OligoCalc. Primers were chosen which had a predicted $T_m$ of 60ºC, and avoided self-complimentarity. For cloning into expression vectors, a non-complimentary 3' sequence was designed to allow ligation at particular cloning sites. Care was taken to maintain the reading frame of the transgene and any subsequent polycistronic elements (such as the IRES-GFP in pCIG). When cloning genes for antisense RNA probes, Taq DNA polymerase alone was used (Promega, Roche). When cloning genes for expression vectors, a mix of Taq (Promega) and Pfu DNA polymerase (Promega) was used. For *Paraxis*, a plasmid containing full-length *Paraxis* (a gift from Suzanne Dietrich) was used as a template. For all other clones a cDNA library made with HH12 whole chick embryo lysates was used.

PCR was performed in an Eppendorf Mastercycler Gradient 5331 thermal cycler, using an appropriate extension time (roughly one minute per kilobase). PCR products were separated using agarose gel electrophoresis, and the appropriate fragment isolated using the QIAquick gel extraction kit (Qiagen, 28704). Fragments for RNA probes were cloned into TOPO cloning vector (Invitrogen). Fragments for RNA probes were prepared by restriction digest and ligated into a linearised plasmid, or cloned using the InFusion PCR cloning kit (Clontech). Plasmids were transformed into XL1 Blue (Stratagene) or DH5α (Invitrogen) competent *E. coli* cells. Clones were analysed by miniprep (Qiagen, Promega) and restriction digest or sequencing. High concentration plasmids (1ug/ul for RNA probes, >5ug/ul for expression vectors) were generated using maxi- or megaprep kits (Qiagen, Invitrogen). DNA concentration was assayed using a Nanodrop 1000 Spectrophotometer (Thermo).

## Morpholino Preparation

Morpholino sequences previously described to target *Pax3* and *Pax7* (Basch and Bronner-Fraser, 2006) were synthesised by Gene Tools, LLC.

## Western

Tissues were lysed in lysis buffer (50 mM Tris pH7.5, 0.27 M sucrose, 1 mM Na-orthovanadate, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 5 mM sodium pyrophosphate, 1% (v/v) Triton-X 100, 0.1% β-mercaptoethanol) with protease inhibitors (Roche), and sonicated to shear genomic DNA. Protein concentration was measured using a Bradford assay. Proteins run on 12%

Bis/Tris NuPAGE gels (Invitrogen) in MOPS (Invitrogen) and transferred to nitrocellulose in transfer buffer (Invitrogen) with 20% methanol. Proteins were detected with antibodies described elsewhere. HRP-conjugated goat secondary antibodies (Abcam, Pierce) were used to detect protein, and subjected to ECL revelation (Pierce).

## PNGase Treatment

Protein N-Glycosidase (PNGase, New England Biolabs) was used to remove α2-5 linked polysialic acid residues from NCAM. Lysates were prepared as normal, denatured, and incubated with the PNGase enzyme overnight at 37ºC.

# Results

Pax genes are important regulators of development. In the paraxial mesoderm, class 1 (*Pax1/9*) and class 3 (*Pax3/7*) Pax genes are important for the correct development of the sclerotome and myotome respectively. Recently it has been suggested that *Pax3* may be also involved in the initial segmentation process in the PSM. We have examined the expression, regulation and function of *Pax3/7* in segmentation.

## Expression of *Pax3/7* in the Chicken Embryo

The early expression pattern of *Pax3/7* in the chick PSM has not been thoroughly explored, with previous attempts focussing on it's role in the epithelial somite during myogenesis (Galli *et al.*, 2008). With this in mind, we first tried to systematically describe the expression pattern of *Pax3* and *Pax7* in the PSM using *in situ* hybridisation.

### Development

*Pax3* is expressed in the primitive streak and lateral neural plate from HH5 (fig. 3.1a). Faint staining is visible in newly forming somites at the 2-3 somite stage (fig. 3.1b), although this has not been confirmed in section. It is also possible that there is some staining throughout the caudal segmental plate at this time.

In more mature embryos, *Pax3* is expressed in the region of the tailbud, the progenitor pool for the PSM and neural tube (fig. 3.1c-e, k). As progenitor

cells in the presumptive dorsal neural tube differentiate, they upregulate *Pax3*, while nascent ventral neural tube and mesoderm downregulate *Pax3* (fig 3.1i,j). In embryos over HH12, *Pax3* expression throughout the A-P axis is maintained, at the lateral edges of the PSM (fig. 3.1d,e). It should be noted that *Pax3* was not detected in definitive intermediate mesoderm or the pronephros. In the rostral PSM, *Pax3* is upregulated in the PSM, including the medial and ventral region (fig. 3.1g). In particular, we observed bands of expression in the rostral PSM, at s-1 or s-2 (fig. 3.1d,e). After somites are formed, *Pax3* is downregulated in the ventral somite, but gradually intensifies dorsally (fig. 3.1f). It should be noted that expression of *Pax3* appears as a rostrocaudal gradient, with highest levels in the most mature somites and lowest in the PSM (Galli *et al.*, 2008). Due to the comparatively low abundance of *Pax3* transcript in the PSM, it is not clear whether its absence in some embryos (e.g. fig. 3.1c) is due to the sensitivity of the *in situ* hybridisation technique.

*Pax7* has a similar expression pattern to *Pax3*, with a few exceptions. It is not expressed at the primitive streak (fig. 3.1m). In neural tissue, is only upregulated at the neural plate border at HH6 and in the dorsal domain after formation of a definitive neural tube (fig. 3.1m-p). It does not appear to have a lateral expression domain in the PSM, but is upregulated along with *Pax3* in the rostral PSM and somites, where its pattern matches *Pax3*. *Pax7* mRNA does not appear to have the rostrocaudal gradient characteristic of *Pax3*, as described previously at the protein level (Galli *et al.*, 2008).

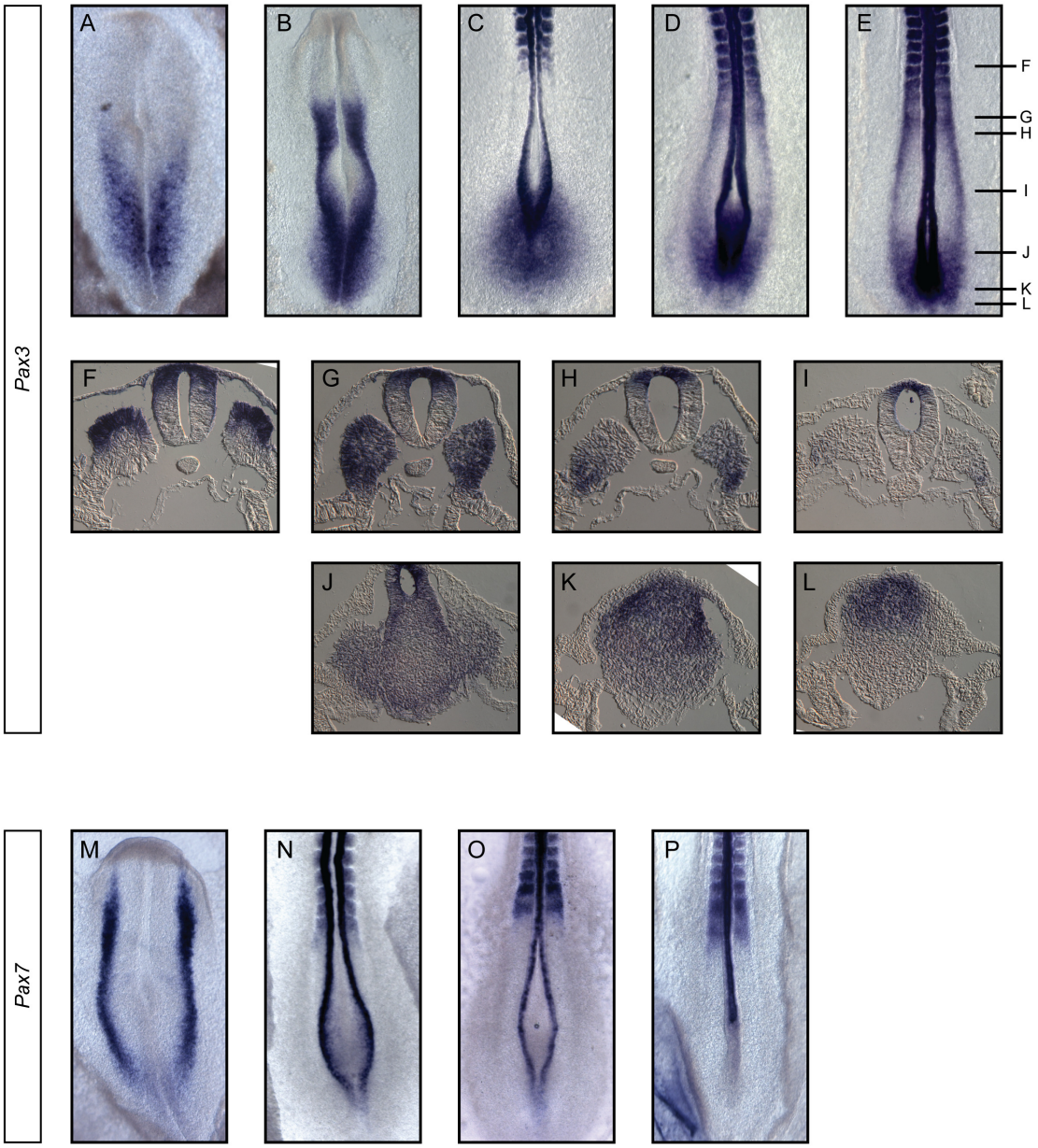


**Figure 3.1: Expression Pattern of *Pax3/7***

A-E: whole mount *in situ* hybridisation for *Pax3* at different stages of development; HH5 (A), HH8 (B), HH9 (C), HH11 (D) and HH12 (E). F-L: transverse cryosections of a HH13 embryo stained for *Pax3* at the levels indicated in (E). M-P: whole mount *in situ* hybridisation for *Pax7* at different stages of development; HH5 (M), HH9 (N), HH11 (O), HH12 (P).

## Potential Cyclic Expression

The pattern of *Pax3/7* expression in the rostral PSM is not even, but has a characteristic banded pattern (fig. 3.1d,e,p). This is similar to several genes which are important for segmentation, such as *EphrinB2* and *Tbx18*. These genes are thought to be outputs of the segmentation clock and exhibit different expression patterns at different phases of the segmentation cycle (Suetsugu *et al.*, 2002; Tanaka and Tickle, 2004). In order to assay whether *Pax3* expression varies throughout the clock cycle, we performed a fix and culture assay to compare PSMs from the same embryo in different phases of the segmentation cycle. Briefly, a caudal chick embro is bisected along the midline to produce two tissue explants whose segmentation clocks are in the same phase. One explant is fixed immediately while the other is cultured a time equivalent to half a segmentation clock cycle (45 minutes in chicken), allowing us to examine how genes change throughout each segmentation cycle (fig. 3.2a). The results were surprising. In most cases (n=4/5), one half of the embryo showed a marked downregulation of *Pax3* expression in the rostral PSM, and even in somites which had been formed at the time of incubation. This happened equally in fixed (n=2) and cultured (n=2) explants (fig. 3.2b,c). It was difficult to identify a clear rostral band in any of the embryos.

The simplest explanation for this result is that Pax3 expression is completely abolished and reinstated during the course of a segmentation cycle. However, this pattern has not been observed in any known output of the

segmentation clock, which is generally characterised by a wavelike expression pattern proceding from caudal to rostral. Additionally, we could not identify an equivalent expression pattern in whole mount embryos. For these reasons, we believe that the disappearance of *Pax3* in half embryos was an artefact of the culture system.

It is possible that in these cases one explant receives more of an axial tissue such as the floor plate or notochord which produces a factor essential for *Pax3* maintenance. We examined the explants by cryosectioning to discover whether one half was receiving more of the notochord, but the results were inconclusive (data not shown).

An alternative approach to describe the expression of *Pax3* throughout the segmentation cycle is to compare it to the expression of a known cyclic gene. The Notch signalling pathway modulator *Lfng* goes through characteristic cycles during the formation of each segment, and potentially provides a means to compare *Pax3* during different phases of the segementation clock. However, for technical reasons we were unable to obtain large enough numbers of these samples to form clear conclusions. The results are described in Appendix 1.

## *Pax3* and cell signalling in the PSM

In studying the expression pattern of *Pax3* in the PSM we have uncovered tantalising clues that it may show dynamic regulation in the rostral PSM. In order to investigate this further, we set out to examine the regulation of *Pax3* by the signalling pathways active in the PSM.

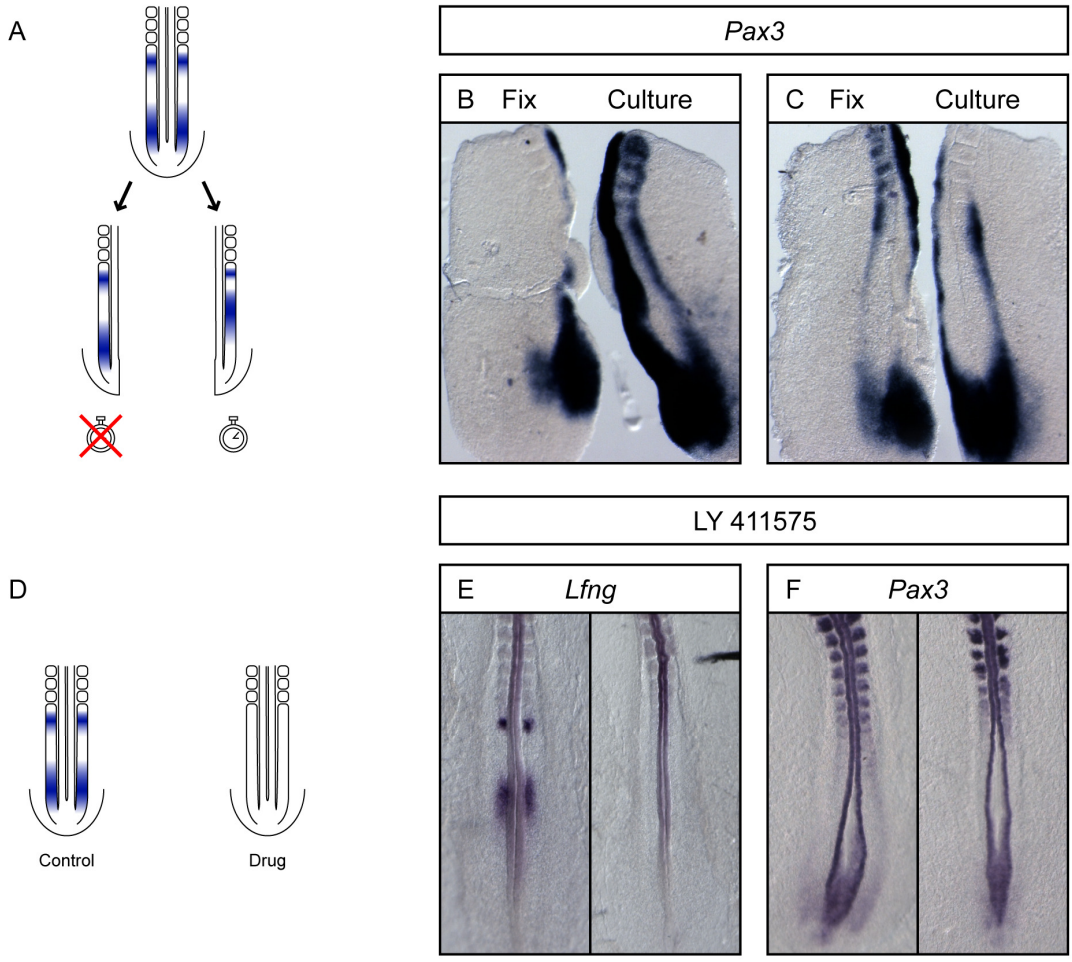


**Figure 3.2: Regulation of Pax3**

A-C: Fix and Culture of *Pax3*. Both halves were cultured for 10 minutes, before one half was fixed and the other cultured for a further 45 minutes. Both were subsequently subjected to parallel whole mount in situ hybridisation (A). In about half of cases (B, n=2/5) the fixed side exhibited downregulated staining while in the rest, the staining on the cultured side was downregulated (C, n=2/5). D-F: effect of the γ-secretase inhibitor LY411575 on Notch target *Lfng* (E, control: n=3, LY: n=3) and *Pax3* (F, control: n=4, LY: n=6) in EC culture over 12 hours.

Notch signalling is an integral part of the feedback loop responsible for generating the periodic segmentation of the PSM, and inhibition of Notch signalling using γ-secretase inhibitors such as LY411575 causes interruption of cyclic gene expression and severely disrupts formation of segment borders. In the chick embryo, this results in the formation of two to six disrupted and non-bilaterally paired segments before segmentation is permanently arrested (Ferjentsik *et al.*, 2009, Gibb, 2009).

Because of the problems assaying *Pax3* in half embryos, we used EC culture to analyse the effect of LY411575 on *Pax3* expression, and found that expression appeared to be correctly upregulated in the segmented region in spite of the loss of Notch signalling (fig. 3.2f, n=6/6). The banded pattern of *Pax3* in the rostral PSM was not discernable, although this may be a result of the abnormal morphology of the paraxial mesoderm. Curiously, while *Pax3* appears to be correctly expressed in the few disrupted segments that are produced after treatment, it does not continue to regress when segmentation ends.

The Wnt signalling pathway is also important in the development of the paraxial mesoderm, and is thought to be involved in the regulation of *Pax3* in the somites (Maroto *et al.*, 1997). We have performed a preliminary investigation of the role of Wnt signalling in *Pax3* expression in the rostral PSM (see Appendix 1).

In summary, we have described the expression of *Pax3/7* in the PSM, and performed a preliminary examination of a potential dynamic expression of *Pax3* in the rostral domain. However, in order to properly understand the role of *Pax3/7* in the process of segmentation, it is necessary to directly examine their function in the PSM.

## Ectopic *Pax3/7*

In order to directly investigate the function of *Pax3/7* in the chicken PSM, we used an *in ovo* electroporation technique. Briefly, DNA is transfected into the epithelial precursors of the paraxial mesoderm in the primitive streak (fig. 3.3). A previous study within the lab (E. Rozenzweig and M. Maroto, unpublished) had established that transfecting the primitive streak with *Pax3* caused disruption of caudal morphogenesis, and disruption of paraxial mesoderm production (fig. 3.3d). A similar problem had been encountered by a group investigating *Meso1* (Watanabe *et al.*, 2007). We solved this problem in a similar fashion by using a tetracycline inducible construct to activate transgene expression after transfected cells had left the primitive streak. The transgene construct was cotransfected with a tetracycline-dependent transcription factor, which could be activated by injection with the tetracycline analogue doxicycline to control the temporal activation of transgene expression (fig. 3.3e).

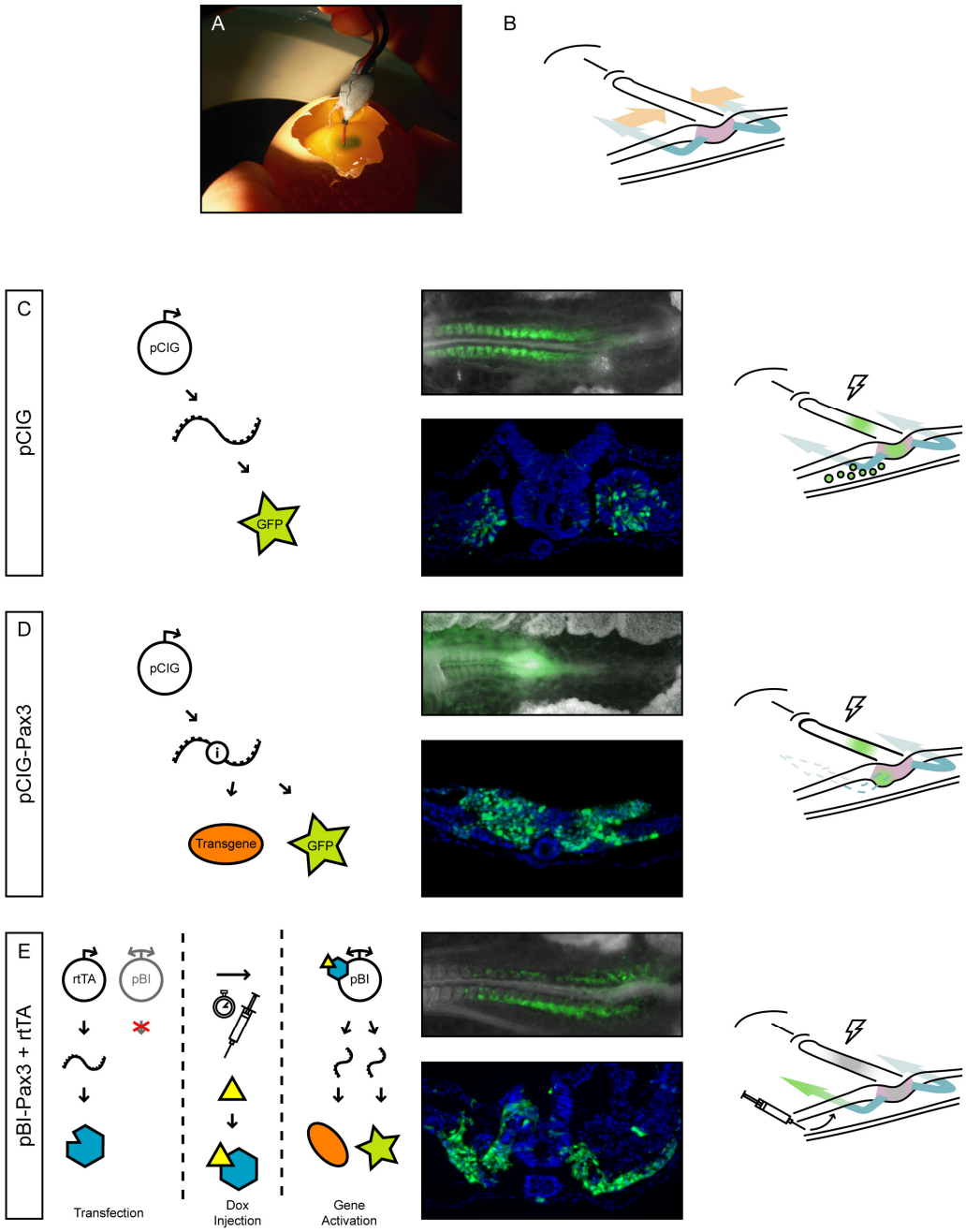


**Figure 3.3: *In Ovo* Electroporation**

A: example of an egg, windowed and injected with DNA solution and having the electrode positioned over the primitive streak. B: schematic diagram of the path of cells entering the primitive streak during gastrulation, and contributing to the paraxial mesoderm (after Gilbert, 2006). C: Targetting of constructs to the paraxial mesoderm by electroporation of the primitive streak. D: Constructs which disrupt the formation of the mesoderm from primitive streak cells, such as pCIG-Pax3 (E. Rosenzweig and M. Maroto, unpublished) prevents study of their effect on the paraxial mesoderm (see also Watanabe *et al.*, 2007). E: Doxicycline inducible constructs can be used to activate gene expression in cells which have transitted the primitive streak, and acquired mesodermal character, allowing study of genes which would otherwise disrupt streak morphogenesis e.g. *Pax3* (Watanabe *et al.*, 2007).

David Wright

## Effect of Pax3/7 on Aggregation and Boundary Formation

We noticed that pBI-Pax3 transfected cells tend to form large clusters, wheras control transfected cells tended to be evenly dispersed throughout the paraxial mesoderm (fig. 3.4a,b, fig. 3.5a). These clumps were not correctly segmented at the level of the first somites, indicating that tonic or upregulated *Pax3* disrupts the formation of intersomitic gaps (fig. 3.4f). However, cells which had been incorporated into somites before induction with doxicycline did not appear to disrupt segmental boundaries.

## Effect of Pax3/7 on Tissue Identity

Exogenous *Pax3* is known from *in vitro* assays to promote differentiation of the paraxial mesoderm into dorsal (dermomyotome) fates (Maroto *et al.*, 1997). To explore the effect of *Pax3/7 in ovo*, we analysed transfected embryos for paraxial mesoderm identity markers by *in situ* hybridisation. *Tbx6* is a marker of PSM identity which is downregulated immediately prior to segmentation by *MesP* (Oginuma *et al.*, 2008, fig. 3.6a). pBI-Pax3 (n=9) and pBI-Pax7 (n=8) both downregulate *Tbx6*, indicating that transfected tissue has lost PSM identity (fig. 3.6b-e). pBI-Pax3 (n=10) and pBI-Pax7 (n=3) also upregulates a marker of dermomyotomal differentiation, *Myf5* (fig. 3.6g-j). Interestingly, only a subset of transfected clusters express *Myf5*. However, neither pBI-Pax3 (n=4) or pBI-Pax7 (n=1) upregulate *Pax1*, the marker of

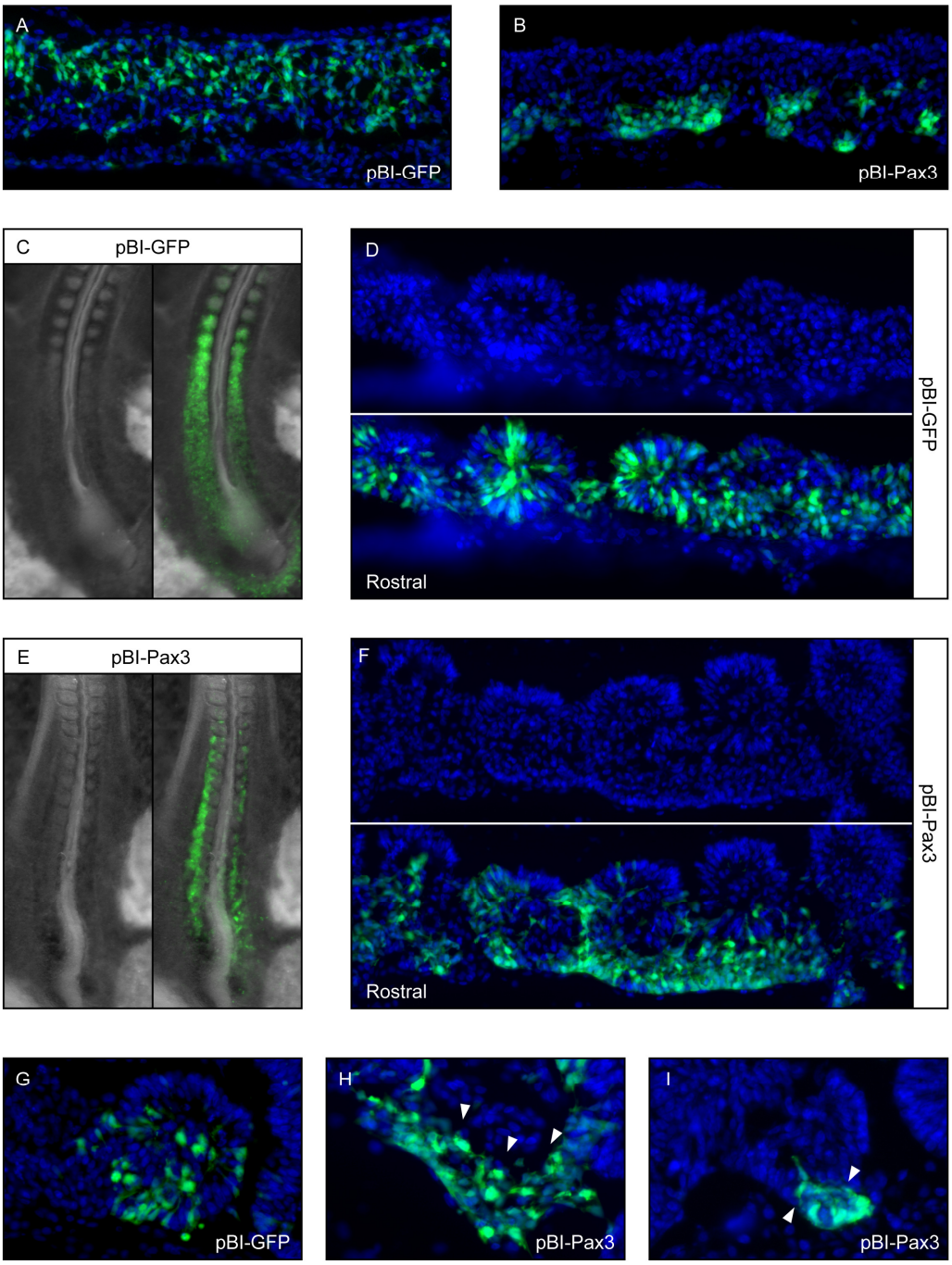


**Figure 3.4: Transfection of Pax3 into the Paraxial Mesoderm**

Transfection of pBI-GFP (A, C-D, G) results in an even distribution of cells throughout the presomitic mesoderm (A), and random contribution to the somites (D, G). Transfection of pBI-Pax3 (B, E-I) results in clustering of transfected cells within the paraxial mesoderm (B). In cases where transcription was induced in the PSM, transfection causes disruption of segmentation and exclusion from somites, wheras in cells which had already been incorporated into somites when transcription was induced, cells appear to remain within the somite (F). In some cases, transfection induces a fissure between transfected and untransfected tissue (H, n=10/15), although in other cases it does not (I, n=11/15).

David Wright

sclerotome differentiation in the ventral somite, supporting the idea that *Pax3/7* specifically drives differentiation of dermomyotome (fig. 3.6k-m). However, it is not clear whether normal *Pax1* upregulation in the sclerotome is inhibited by overexpression of *Pax3/7*.

## Effect of Pax3/7 on Cell Adhesion

When studying the tissue architecture of transfected PSM, it was clear that pBI-Pax3 and pBI-Pax7 were having a dramatic effect, as noted previously (fig. 3.4). Because pBI-Pax3/7 were implicated in precocious differentiation of the tissue, and there is an established antero-posterior maturation of the PSM in terms of cell adhesion and extracellular matrix, culminating in a mesenchymal-to-epithelial transition (Duband *et al.*, 1987), we investigated the effect of pBI-Pax3/7 on markers of adhesion, matrix, and cytoskeletal organisation.

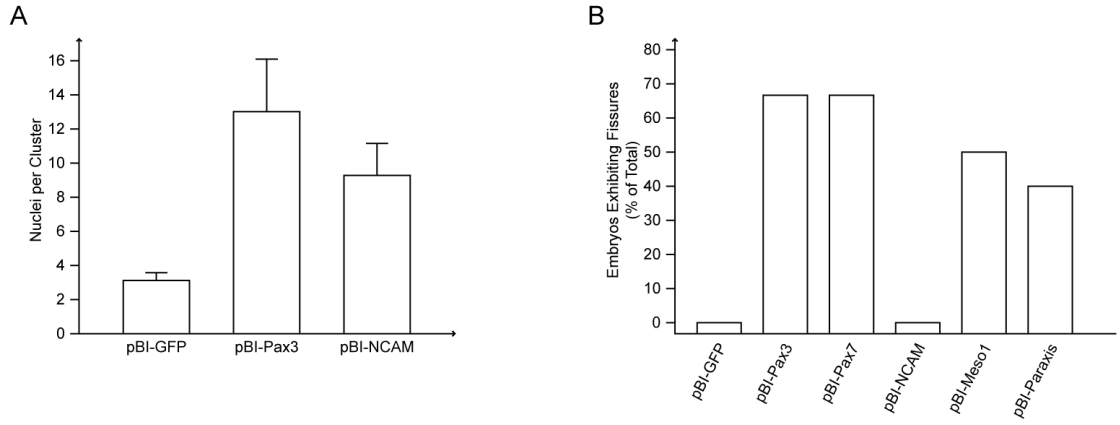


**Figure 3.5: Cluster size and Fissure Frequency**

A: Cluster size in transfected tissue. Representative sections of pBI-GFP (3 embryos), pBI-Pax3 (3 embryos) and pBI-NCAM (1 embryo) were analysed to describe how many cells were present in each cluster. B: Fissure formation in transfected tissue. Presence of fissures was investigated in pBI-GFP (0/11), pBI-Pax3 (10/15), pBI-Pax7 (6/9), pBI-NCAM (0/3), pBI-Meso1 (2/4), pBI-Paraxis (4/10).

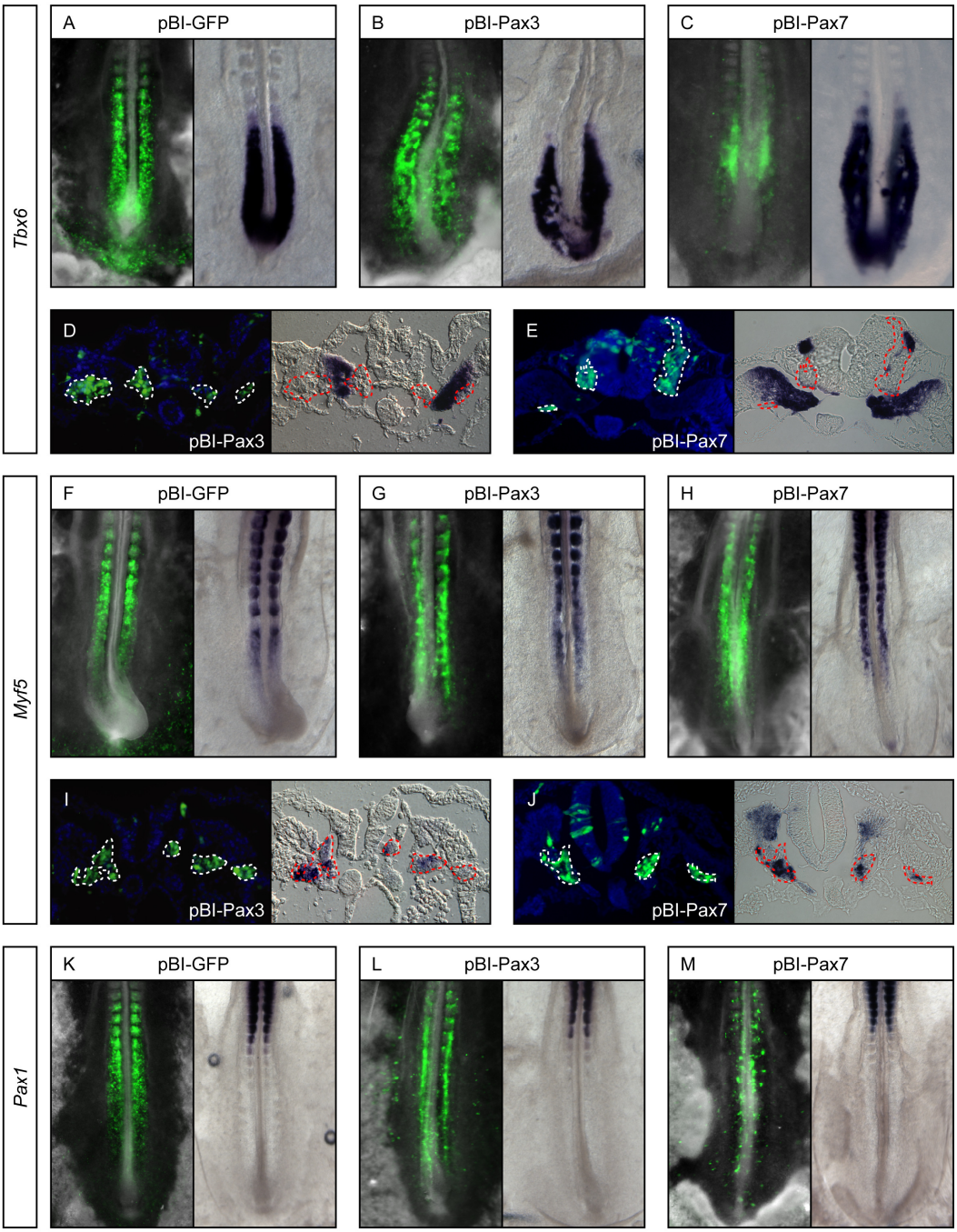


**Figure 3.6: *Pax3/7* and Tissue Identity**

A-E: PSM identity is assayed by whole mount in situ hybridisation of *Tbx6*. Transfection with pBI-GFP does not affect expression of *Tbx6* (A, n=9), while transfection with *Pax3* (B, D, n=9) and *Pax7* (C, E, n=8) cause a downregulation of *Tbx6*. F-J: *Pax3/7* cause upregulation of the myogenic factor *Myf5*. pBI-GFP does not induce *Myf5* (F, n=10) while transfection with pBI-Pax3 (G, I, n=10) and pBI-Pax7 (H, J, n=3) cause a profound upregulation of *Myf5* throughout the PSM. K-M: Pax3/7 do not induce sclerotome, assayed by the sclerotomal marker *Pax1*. pBI-GFP (K, n=5), pBI-Pax3 (L, n=4) and pBI-Pax7 (M, n=1) do not upregulate the sclerotomal marker *Pax1*.

David Wright

NCAM is an important adhesion molecule which is upregulated as the PSM matures, and maintained at high levels in the dermomyotome. It is also known that *NCAM* is under transcriptional control of Pax genes in various developmental contexts (Chalepakis *et al.*, 1994), and that ectopic *Meso1* upregulates NCAM in the anterior PSM via *Pax2* (Watanabe *et al.*, 2007).

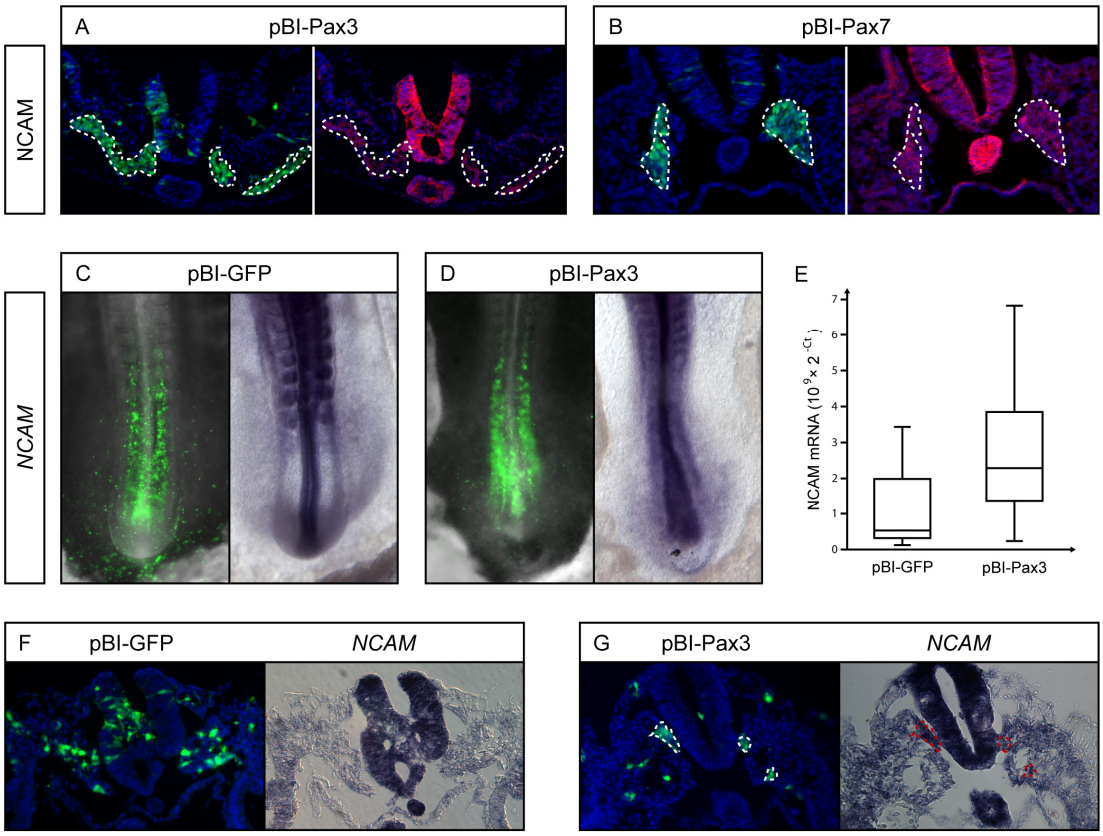


**Figure 3.7: *Pax3/7* cause upregulation of NCAM**

A-B: pBI-Pax3 (A, n=4) and pBI-Pax7 (B, n=5) both induce upregulation of NCAM protein. C-E: Ectopic *Pax3* upregulates *NCAM* at the mRNA level. Whole mount *in situ* hybridisation of transfected embryos shows upregulation in pBI-Pax3 (D, n=4) or pBI-Pax7 (G, n=3) transfected embryos, but not pBI-GFP transfected embryos (C, F, n=3). E: analysis of mRNA levels in pBI-GFP (n=7) and pBI-Pax3 (n=4) transfected PSMs by qRT-PCR shows an upregulation of *NCAM* mRNA.

NCAM is clearly upregulated by pBI-Pax3 (n=4) and pBI-Pax7 (n=5) at the protein level (fig. 3.7a,b), however it is possible that this reflects a differential distribution of protein in transfected cells. To explore whether pBI-Pax3 was causing transcriptional activation of *NCAM* we assayed mRNA levels. *NCAM* appeared to be upregulated by pBI-Pax3 (n=4; fig. 3.7c,d) and pBI-Pax7 (n=3; fig. 3.7f,g) by *in situ* hybridisation, however because of the presence of a background signal in the PSM, it was not possible to be certain. For this reason, we generated cDNAs made from transfected PSM tissue and analysed them by qRT-PCR, supporting the notion that *NCAM* transcription is upregulated following *Pax3* transfection (fig. 3.7e).

The effect of pBI-Pax3/7 on N-cadherin expression and the cytoskeleton are discussed in Appendix 3.

## Extracellular Matrix

The extracellular matrix is important in segmentation, the formation of morphological boundaries and correct epithelialisation of newly formed somites. Fibronectin has been noted as being especially important (Rifes *et al.*, 2007), with proteases that disrupt the fibronectin network also preventing segmentation. Fibronectin is secreted as soluble monomers that are assembled into fibrils in a process which involves fibronectin's cellular receptors, the integrins. A proportion of the fibronectin subunits are thought to originate from the ectoderm.

The effect of pBI-Pax3 (n=7) and pBI-Pax7 (n=2) on fibronectin is not clear cut. Where transfected tissue has become discontiguous with non-transfected tissue, fibronectin clearly demarcates the boundary (fig. 3.8b, d). Even in

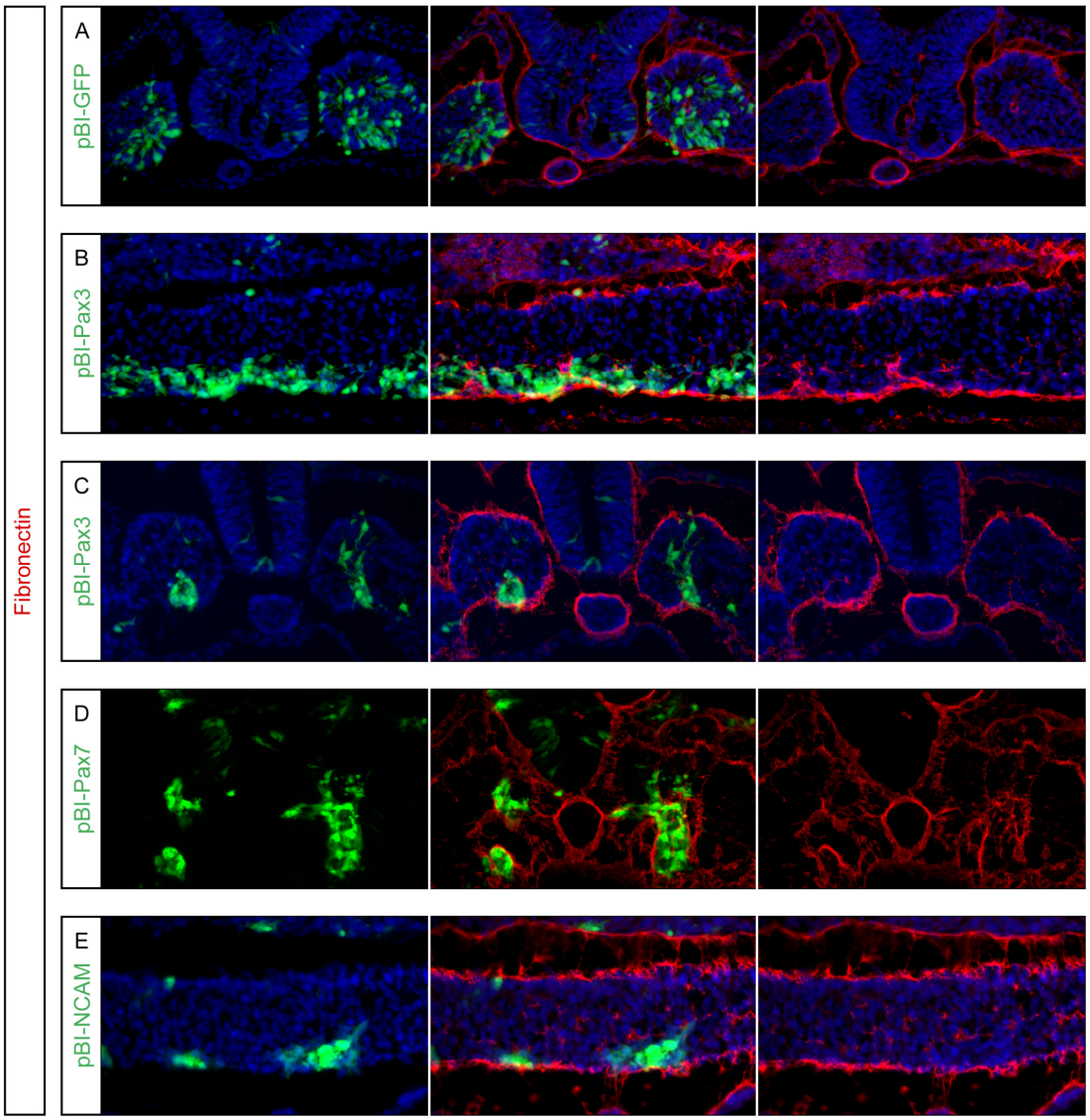


**Figure 3.8: Effect of *Pax3/7* on Fibronectin**

A-D: Transfection with pBI-Pax3 (B, shown in longitudinal section, n=7) and pBI-Pax7 (D, n=2) results in the upregulation of fibronectin around the transfected area, as compared to pBI-GFP (A, n=4). The extent of fibronectin appears to be linked to the degree of separation from the untransfected paraxial mesoderm (C). In contrast, transfection with pBI-NCAM did not induce fissure formation or fibronectin assembly (E, shown in longitudinal section, n=2).

cases where there is not a clear morphological separation between the two domains, some fibronectin seems to accumulate at their interface (fig. 3.8c). It is not clear whether fibronectin fibrillogenesis is actively induced at the pBI-Pax3/7 overexpression boundary, or whether fibrillogenesis occurs opportunistically in fissures between transfected and non-transfected tissue.

It should also be considered that because short fibrils of fibronectin are expressed throughout the anterior PSM (Duband *et al.*, 1987), and mock transfected cells are distributed throughout the tissue rather than forming clusters, it is difficult to definitively rule out the possibility that transfection upregulates fibronectin (fig. 3.8a).

We also examined whether fibronectin was upregulated around pBI-NCAM transfected clusters, as this recapitulates part of the pBI-Pax3/7 overexpression phenotype, and found that clusters were not associated with upregulated fibronectin (n=2, fig. 3.8e). However, as we could not detect gaps between transfected and untransfected tissue, this does not address whether fibronectin is forming opportunistically in *Pax3/7* electroporated embryos.

While we cannot rule out the regulation of *fibronectin* expression by pBI-Pax3/7, the endogenous mRNA expression pattern (Rifes *et al.*, 2007) and distribution of fibronectin outside transfected clusters suggests that fibrillogenesis is an indirect response to transfection. One mechanism

through which pBI-Pax3/7 could be causing fibrillogenesis is through upregulating cellular receptors for fibronectin, the integrins. We performed a preliminary assay for the integrin $\alpha$5 subunit, which is the most well described isoform in this tissue, but could not detect an upregulation by pBI-Pax3 (not shown).

Ovexpression of *Pax3/7* has a profound effect on tissue architecture in the paraxial mesoderm. Transfected cells cluster, separate from untransfected tissues, and do not segment properly. The clustering appears to be a result of upregulation of cell adhesion molecules such as NCAM. Transfection also appears to activate parts of the epithelialisation process, such as actin polymerisation and formation of a fibronectin matrix, but prevents the formation of a polarised somitic epithelium.

## Transcription Factors in the PSM

In addition to factors required for tissue identity, such as *Tbx6*, a complex network of transcription factors is active in the rostral PSM which are thought to play an important role in the process of segmentation. In addition to *Pax3* and *Pax7*, the myogenic factor *Myf5* is also expressed with a dynamic expression pattern in the rostral PSM. *MesP* genes, and their chick orthologues *Meso*, are expressed at the very rostral PSM, and are associated with *Pax2* expression in chick. *Paraxis* is also known to be important in the formation of epithelial somites. In most cases, the interactions between these

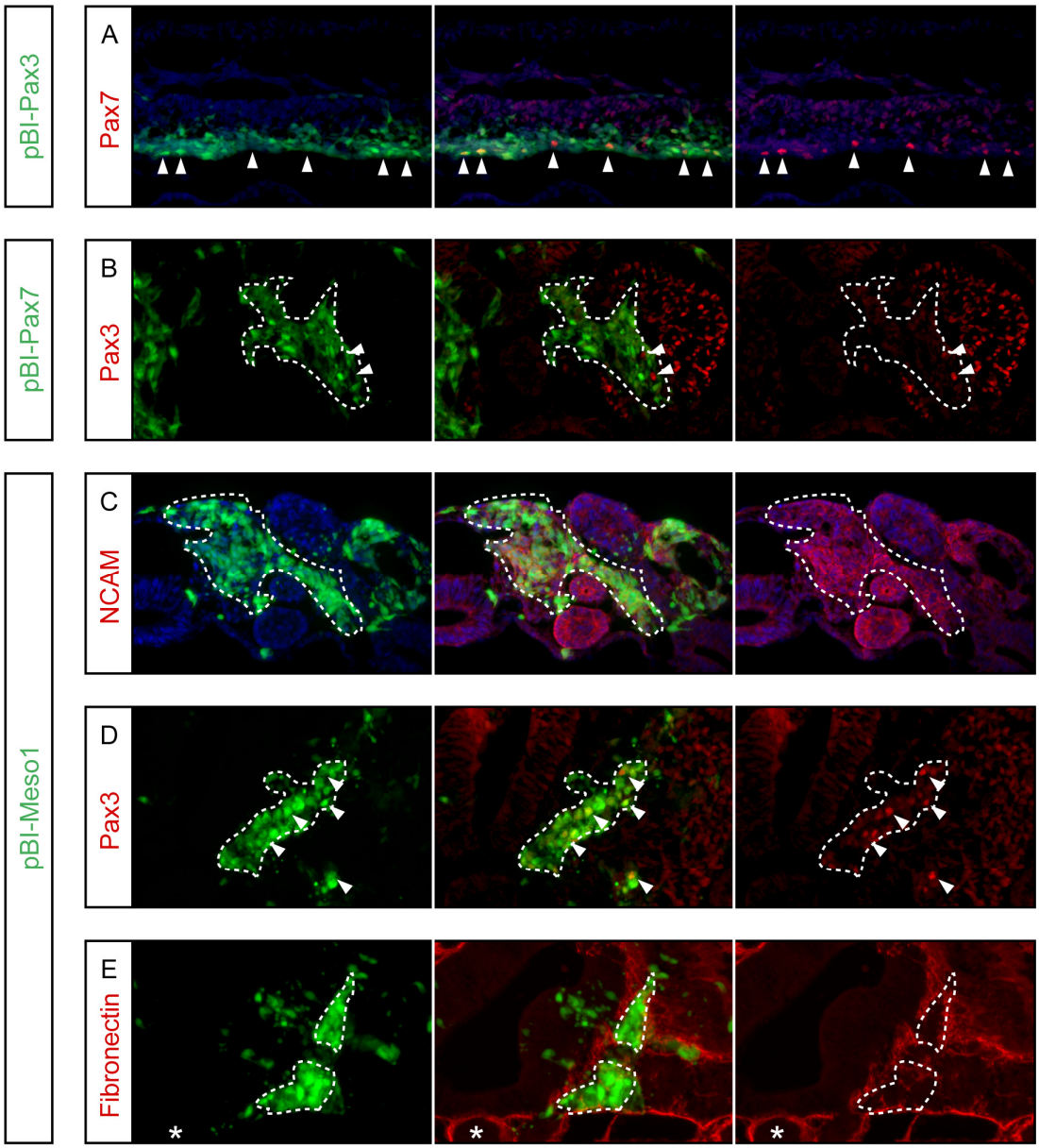


**Figure 3.9: Effect of Ectopic *Pax3/7* on *Pax3*, *Pax7*, and *Meso1***

A-B: Immunological detection of Pax7 protein in pBI-Pax3 transfected PSM (A, shown in longitudinal section, n=1/2) and Pax3 protein in pBI-Pax7 transfected PSM (B, n=2). C-E: Effect of transfection with pBI-Meso1. NCAM is upregulated by transfection with pBI-Meso1 (C, n=2) as described previously (Watanabe *et al.*, 2007). Transfection with pBI-Meso1 causes upregulation of Pax3 in a subset of cells (D, n=3). pBI-Meso1 appears to have a minor effect on fibronectin (D, n=2).

factors is poorly defined. For this reason, we decided to investigate their effects on each other using our PSM transfection assay.

## *Pax3* and *Pax7*

The effect of *Pax3* and *Pax7* on each other is an interesting effect, as they appear to have an overlapping expression pattern in the rostral PSM (fig. 3.1) but their domains become exclusive as differentiation continues (Galli *et al.*, 2008).

The effect of pBI-Pax3 on *Pax7* and vice versa is not straightforward. In general, the effect appears to be a downregulation, with transfected cells exhibiting lower immunoreactivity for *Pax3* (n=2; fig. 3.9b) or *Pax7* (n=2; fig 3.9a) than untransfected cells. However, in both cases a minority of cells exhibited upregulation of *Pax3* (n=1/2) or *Pax7* (n=1/2). In embryos transfected with pBI-Pax3, *Pax7* was upregulated within the transfected domain (fig. 3.9a, arrowheads); in pBI-Pax7 transfected embryos, cells expressing high levels of *Pax3* were occasionally detected outside the boundary of the transfected region (fig. 3.9b, arrowheads).

## *Meso1* and *Pax2*

The transcription factor *Meso1* is dynamically regulated by Notch signalling (Yasuhiko *et al.*, 2008), and is thought to contribute to NCAM regulation in the anterior PSM via *Pax2* (Watanabe *et al.*, 2007). Because of the similarity between the phenotype of pBI-Meso1 and pBI-Pax3/7, we examined the effect of pBI-Meso1 expression further. Transfection with pBI-Meso1 seemed to

closely resemble overexpression of pBI-Pax3/7, with cells forming clusters, however unlike pBI-Pax3/7 the border of transfected tissue was often uneven and fissure formation is less common (fig. 3.5b). As described previously, pBI-Meso1 (n=2; fig. 3.9c) and pBI-Pax2 (n=1; not shown) increase NCAM expression. We also noted that clusters of pBI-Meso1 appeared to be associated with increased fibronectin (n=2; fig. 3.9e), although transfected tissue did not form fissures or even clear borders with untransfected tissue.

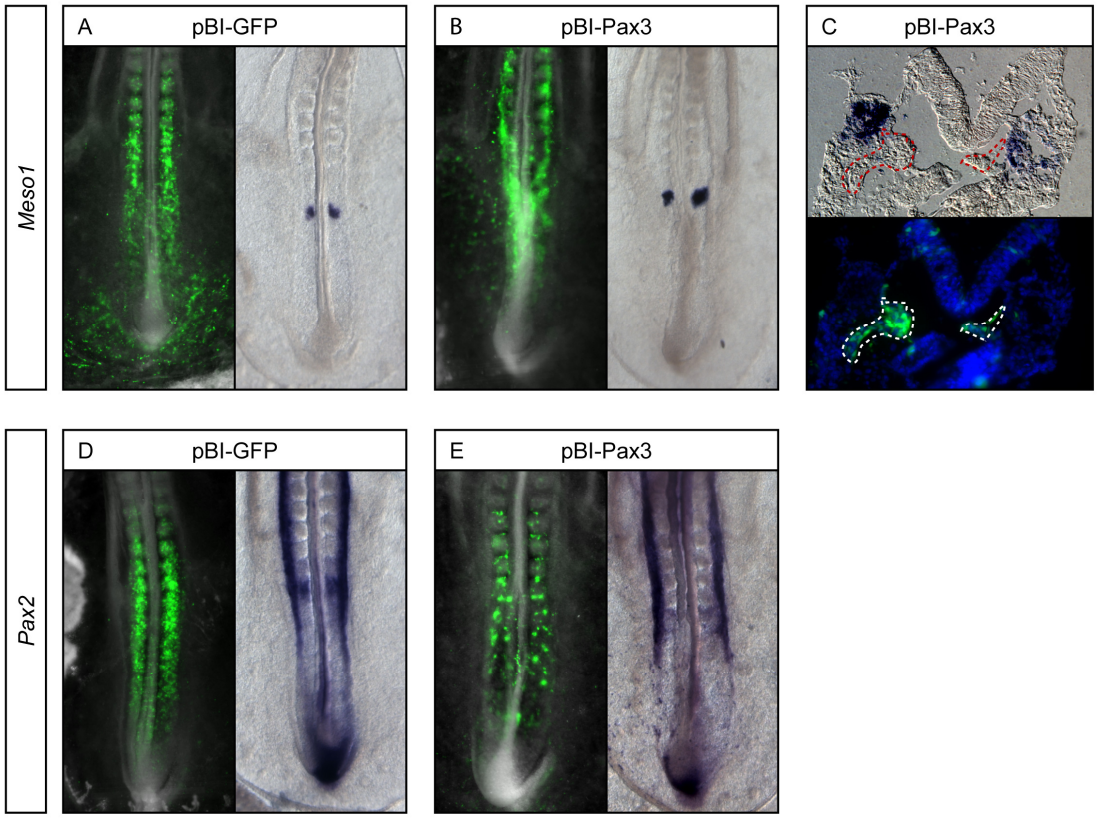


**Figure 3.10: Effect of Ectopic *Pax3* on expression of *Meso1* and *Pax2***

A-C: effect of ectopic *Pax3* on *Meso1*. Transfection with pBI-GFP does not affect *Meso1* (A, n=3) while pBI-Pax3 disrupts *Meso1* expression (B, n=5), verified by cryosection (C). D-E: effect of ectopic *Pax3* on *Pax2* expression. Neither pBI-GFP (D, n=3) or pBI-Pax3 (E, n=9) have any effect on the expression of *Pax2*.

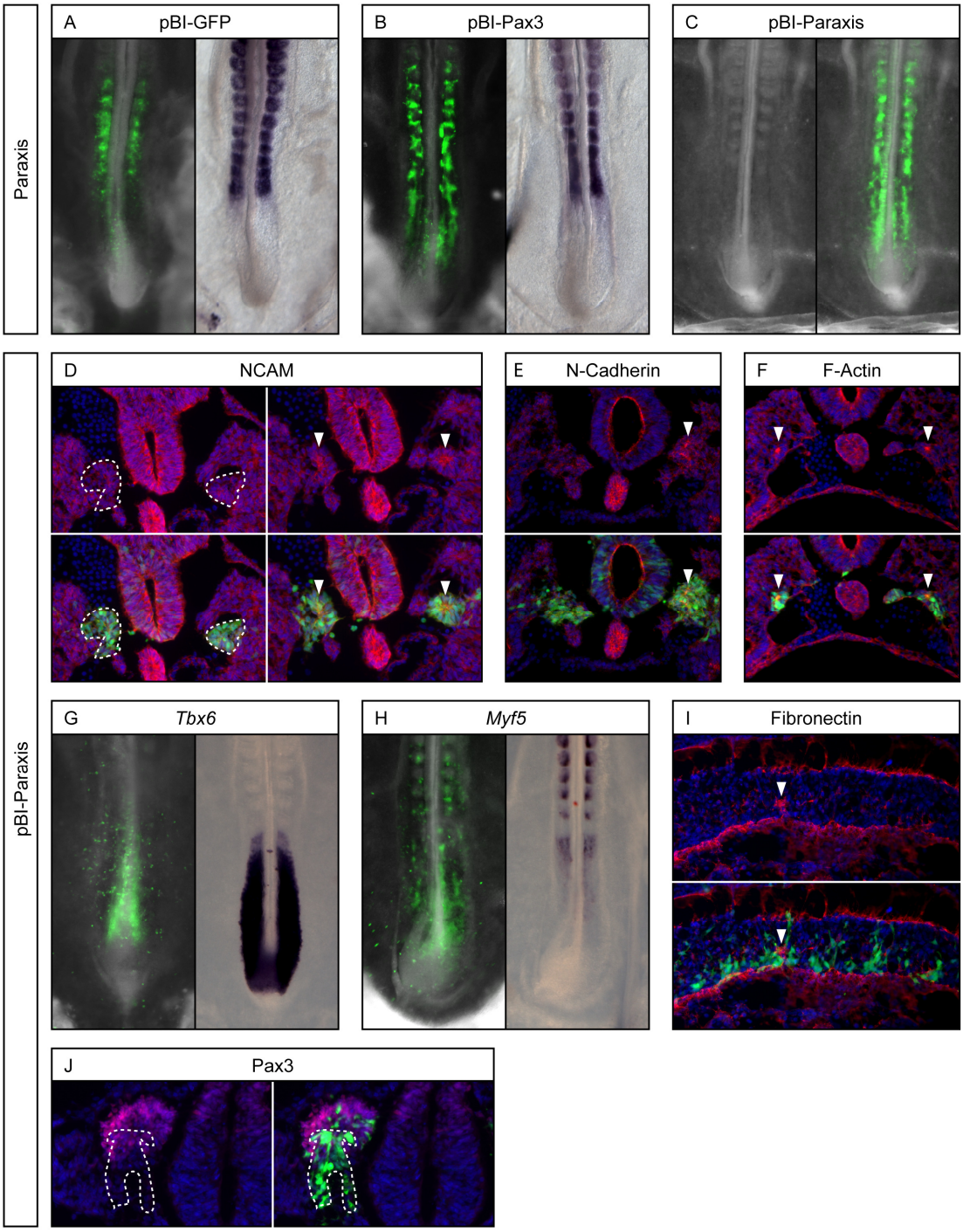


**Figure 3.11: Interactions between *Pax3/7* and *Paraxis***

A-B: Effect of transfection on *Paraxis* expression assayed by *in situ* hybridisation. *Paraxis* is not affected by pBI-GFP (A, n=3) or pBI-Pax3 (B, n=6) transfection. C-J: Effect of transfection of pBI-Paraxis on PSM structure and identity. NCAM is not upregulated by pBI-Paraxis (D, n=5), but shows organisation into rosette-like structures (n=4/5). N-cadherin (E, n=4) and F-actin (F, n=3) are not upregulated, but reorganised by pBI-Paraxis transfection. pBI-Paraxis did not have any detectable

**cont. overleaf**

We also investigated the relationship of *Meso1* and *Pax3*. Interestingly, a subset of pBI-Meso1 transfected cells upregulate Pax3 (n=3, fig. 3.9d, arrowheads). This effect could not be reliably detected upon transfection with *Pax2* (not shown). Conversely, transfection with pBI-Pax3 downregulates *Meso1* mRNA (n=5, fig. 3.10b, c). Pax3 did not upregulate *Pax2* mRNA in the PSM (n=9, fig.3.10e), but we cannot rule out a downregulation.

## *Paraxis*

*Paraxis* is involved in epithelialisation of the paraxial mesoderm. Like *Pax3* and *Pax7*, it is upregulated in the anterior PSM throughout the dorsoventral axis, before becoming restricted to the dermomyotome, and is known to be important in the epithelialisation of somites.

We first investigated whether pBI-Pax3 had any effect on *Paraxis*, but could find no abnormal upregulation of *Paraxis* by *in situ* hybridisation (n=6, fig. 3.11a, b). We then cloned full length *Paraxis* into the doxicycline-inducible vector, and transfected it into the PSM (fig. 3.11c).

**Figure 3.11: Interactions between *Pax3/7* and *Paraxis* cont.**

effect on *Tbx6* (G, n=2) or *Myf5* (H, n=2) expression, assayed by *in situ* hybridisation. pBI-Paraxis does mild induce fissure formation (D, arrowheads) and fibronectin assembly (I, n=2). pBI-Paraxis does not have a detectable effect on Pax3 protein expression. (J, n=4).

At a whole mount level, pBI-Paraxis transfected cells appeared to exhibit a similar degree of clumping to pBI-Pax3/7, however transfection did not appear to cause disruption of segmentation. Clusters occasionally separated from the PSM, although at a lower rate than pBI-Pax3 transfected embryos (fig. 3.5b).

Neither NCAM (n=5; fig. 3.11d) nor N-cadherin (n=4; fig. 3.11e) appear to be upregulated by pBI-Paraxis, although in many cases it was possible to observe a rosette-like arrangement of NCAM (n=4/5; fig 3.11d, arrowheads), and apical localisation of N-cadherin (n=4/4; fig 3.11e, arrowheads) and F-Actin (n=3/3; fig 3.11f, arrowheads), suggesting that *Paraxis* regulates aspects of somitic epithelialisation. Fibronectin also appears to be slightly upregulated around the electroporated region, but not to the same degree as pBI-Pax3/7 (n=2, fig. 3.11i).

We tested whether *Paraxis* had an effect on PSM identity and maturation, and found no effect on either *Tbx6* (n=2) or *Myf5* (n=2) when transfected with pBI-Paraxis (fig. 3.11g, h). No effect could be detected on *Pax3* expression (n=4, fig. 3.11j).

The genetic networks controlling the maturation of the rostral PSM, somite formation and differentiation of the distinct lineages of the paraxial mesoderm are extremely complex. *Pax3/7* do not function in isolation, but affect and are affected by reciprocal networks of genes including other Pax genes, *Meso*,

*Paraxis* and *Myf5*. Detailed future work will be required to fully elucidate the function of these gene networks, and their relationship to morphogenesis and differentiation.

# Discussion

We have analysed the expression and function of *Pax3/7* in the chick PSM. As a result, we have described a previously unstudied domain of expression in the rostral PSM and performed a preliminary study of its regulation. Functional analysis of *Pax3/7* by transfection revealed that ectopic expression of these genes causes changes in the tissue morphology, identity, and adhesion of transfected tissue. Finally, we have tried to put these genes in the context of the genetic network active in the rostral PSM.

## Pax3 Expression Domains

The expression patterns of *Pax3* and *Pax7* in the rostral PSM have not been investigated in as much detail as their role in the maturing somites. In the mouse, the earliest expression of *Pax3* in the PSM is in the rostral PSM immediately before segmentation. While *Pax7* follows this pattern in the chick, *Pax3* has more complex distribution, with domains at the caudal end of the embryo and in the lateral PSM.

It is important to note that *Pax3* exhibits a rostrocaudal gradient of expression in the paraxial mesoderm, as has previously been observed (Galli *et al.*, 2008). This is of great relevance in the light of the dose dependence of *Pax3* function (Zhou *et al.*, 2008; Bajard *et al.*, 2006; Chalepakis *et al.*, 1994). This has also made examining staining in the PSM more complicated, as in some embryos the level of signal is near the threshold of detection. We believe that

this may be the source of many of the artefacts observed in our half embryo system. (fig. 3.2, appendix 1).

## Caudal Mesoderm and Tailbud

*Pax3* is detected in the caudal mesoderm and tailbud in a domain which has not been described in mouse. Interestingly, this domain of expression is detected in both primary (primitive streak mediated) and secondary (tailbud mediated) body development. The presence of *Pax3* in this domain is particularily interesting, as transfection with ectopic Pax3 disrupts gastrulation (M. Maroto and E. Rosenzweig, unpublished observations). Its expression in the tailbud appears to include the dorsal posterior tailbud, a region in the tailbud which contains the long-term stem cell population for the paraxial mesoderm (McGrew *et al.*, 2008), although this will need to be confirmed with more careful examination.

## Lateral Presomitic Mesoderm

A domain of *Pax3* expression in the lateral PSM is detected in embryos above HH11. It is known that there is a mediolateral identity within the paraxial mesoderm, and that medial PSM is required for segmentation of the lateral domain (Freitas *et al.*, 2001), although genes with a mediolateral distribution pattern have not been identified. However, it seems unlikely that Pax3 has a role in driving the mediolateral identity of the PSM as its expression does not robustly appear until somitogenesis is underway. This temporal pattern may represent changes in the signalling environment of the PSM as the embryo matures.

## Rostral PSM

The *Pax3* expression domain in the rostral PSM is particularily interesting. For a short period of time, from approximately S-2 to S2, *Pax3* expression is upregulated throughout the dorsoventral and rostrocaudal axis. This pattern maintains its position relative to the first segmental boundary, and thus appears to regress down the body axis as somitogenesis procedes. Along with staining in the lateral PSM, this expression domain seems to definitively appear at around HH11. It also seems to exhibit a mediolateral gradient of intensity, with strongest expression at the lateral edge of the paraxial mesoderm.

In this domain, *Pax3* has a banded pattern, with expression upregulated along the mediolateral axis to near the midline at S-2, partially downregulated (especially in the medial PSM) at S-1, and upregulated again at S0. This banding pattern is reminiscent of genes such as *Tbx18* or *EphA4*, although these genes exhibit a banding pattern at half-somite intervals, while *Pax3* appears to form bands that cover a whole somite domain. This pattern of expression domains is very similar to the expression pattern of *Myf5*, which is also expressed at comparatively low levels in the chick PSM (Kiefer and Hauschka, 2001).

# Regulation

## Cell signalling

The regulation of this rostral domain domain is, as yet, obscure. Its graded mediolateral expression pattern suggest that it might be regulated positively by lateral signals such as BMPs from the lateral plate, or low concnetrations of medial signals such as *SHh*. However, the spatial regulation of *Pax3* in the neural tube does not change at this level on the AP axis, suggesting that regulation may be intrinsic to the PSM. An interesting avenue of investigation would be to examine whether the factors which control anteroposterior development of the paraxial mesoderm, such as *Wnt3a*, *FGF8* and retinoic acid, or intrinsic signalling within the PSM such as Notch, affect the expression of *Pax3* in the rostral PSM. The potential connection between *Pax3* and *Snail2* (see below and Appendix 1), a transcription factor regulated by Wnt and FGF signalling, make these pathways particularily interesting candidates for the regulation of *Pax3*.

Our results tentatively suggest that Notch may not be important for rostrocaudal patterning of *Pax3*, and that Wnt signalling may be important in the regulation of *Pax3* (see Appendix 1). It should also be noted that in targetting casein kinase, we target only canonical Wnt signalling, and do not discriminate between the various Wnts acting on the paraxial mesoderm (Linker *et al.*, 2005). The role of FGF in the regulation of *Pax3* remains to be addressed.

Since this pattern appears to be fixed relative to next forming segment boundary, each presumptive segment presumably undergoes a cycle of *Pax3* activation, inactivation and reactivation before undergoing segmentation. Alternatively, *Pax3* may exhibit a wave-like expression pattern comparable to other cycling genes such as *Lfng*, where it's expression domain moves along the paraxial mesoderm. Our first approach to investigate the effect of *Pax3* on the PSM using a fix-and-culture technique was hampered by technical problems. In most cases, no *Pax3* was detected at all, even in somites which had formed before culture had started. This did not appear to correlate with either culture time or laterality of explants.

One possibility is that this discrepancy may be caused by asymmetric inheritance of a source of cell signalling, such as the notochord. It is known that notochord ablation has an effect on the pace of the segmentation clock via *SHh* (Resende *et al.*, 2010). Mouse mutants for *SHh* exhibit ventralised *Pax3* (Borycki *et al.*, 1999) suggesting *SHh* negatively regulates *Pax3*, however in vitro experiments in chick suggested a positive relationship (Maroto *et al.*, 1997). Alternatively, other signalling molecules, or indeed other signalling centres, may be responsible for the discrepancy.

In order to remove the effect of asymmetric inheritance of axial structures, we attempted to assay changes to *Pax3* expression relative to the oscillating gene *Lfng* (see Appendix 1). In this experiment, explants are not cultured so

inheritance of the axial tissues should not effect *Pax3* expression. However, we continued to have difficulty staining for *Pax3*.

For this reason, we believe that the very low abundance of *Pax3* mRNA is responsible for the problems in half embryo staining. We suggest that future experiments should include untreated explant pairs stained in parallel for *Pax3* as a control.

## Snail

*Snail1* and *Snail2* are a pair of transcription factors associated with mesenchymal cell behaviours (Barrallo-Gimeno and Nieto, 2005). Interestingly, *Snail1* in mouse and *Snail2* in chicken exhibit a cyclic expression pattern in the PSM. As part of this expression pattern, *Snail2* is expressed as a rostral band in the PSM during part of its cycle. It has been suggested that in order for the paraxial mesoderm to undergo mesenchymal-to-epithelial transition during segmentatation, *Snail2* must be downregulated (Dale *et al.*, 2006). Since *Pax3* is associated with an epithelial identity in the mature paraxial mesoderm, we considered whether *Pax3* might be antagonistic with *Snail2* in the chick embryo, and that the rostral band of *Snail2* might coincide with the low-*Pax3* band in the rostral PSM.

Surprisingly, examining the expression pattern in explants of the same embryo revealed that *Snail2* appeared to be expressed at the level of the caudal *Pax3* band, rather than the low-*Pax3* domain. However, numbers for

this analysis were very low, and will require verification. Even if the mRNA domains were coexpressed, *Snail2* may still be responsible for the subsequent downregulation of *Pax3* due to latency in transcription, resulting in the transient downregulation of *Pax3* at S-1. The functional relationship between *Snail2* and *Pax3* in the rostral PSM could be investigated further using our transfection techniques.

## Ectopic *Pax3/7*

In addition to our analysis of the expression and regulation of *Pax3/7* in the PSM, we have also developed an electroporation based transfection technique to study their function. As with previous studies of factors involved in segmentation (Watanabe *et al.*, 2007) simply transfecting with *Pax3/7* disrupts gastrulation, so we have adopted a Tet-on inducible system.

It should be clearly noted that much of our analysis was restricted by the limited number of samples available, and in many cases numbers are not sufficient to draw firm conclusions. In particular, we have assumed that *Pax7* produces similar morphological effects to *Pax3*, although numbers are often too low to draw conclusions about the function of *Pax7* in it's own right. Also, when investigating the effect of transgenes on endogenously expressed markers such as NCAM, using the empty pBI-GFP plasmid as a transfection control was not appropriate due to the difficulty in identifying effects on a mixed transfected/non-transfected cell population. In contrast, when using whole mount *in situ* hybridisation to analyse transfected embryos, using a pBI-

GFP transfected control is important to eliminate the possibility that riboprobes may be detecting the expression plasmid.

Transfection of pBI-Pax3/7 produces an interesting range of effects. It alters cell identity, promotes cell aggregation, activates the cell adhesion molecule NCAM, alters the cytoskeleton, and affects the fibronectin matrix.

## Identity

As an important marker of the myogenic pathway, it seems obvious that pBI-Pax3 and pBI-Pax7 will regulate cell identity. It is interesting to note, however, that PSM identity is suppressed, with transfected cells losing *Tbx6* expression. In untreated embryos, *Tbx6* is lost as each somite is formed. While *MesP2* is important for rapid downregulation of Tbx6 protein, it is not clear what mediates the rapid loss of *Tbx6* mRNA expression at the newly formed somite boundary. While the expression pattern of *Pax7* may be consistent with a role the downregulation of *Tbx6*, the lateralised expression of *Pax3* in the rostral PSM is not. Nevertheless, it is interesting that ectopic *Pax3/7* drives downregulation of *Tbx6*.

Ectopic *Pax3/7* also drives expression of the myogenic marker *Myf5* in a subset of transfected cells. This recapitulates the suggestion that *Pax3* can regulate *Myf5* in some contexts (Maroto *et al.*, 1997, Bajard *et al.*, 2006). This is also the first time that Pax7 has been shown to regulate *Myf5*.

The relationship between these three factors and each other appears to be complex. While in general *Pax3* and *Pax7* appear to mutually exclude each other, tissue transfected with pBI-Pax3 occasionally produces cells with a very high expression of *Pax7*. In contrast, pBI-Pax7 was not observed upregulating *Pax3*. Similarily, it is interesting that *Myf5* is only activated in a subset of pBI-Pax3/7 transfected cells, although the resolution of *in situ* hybridisation is not sufficient to investigate this in detail. Tissue transfected with pCIG-Myf5 also exhibits a minority of cells which express *Pax3* (see Appendix 4).

We also investigated whether pBI-Meso1 could upregulate *Pax3/7*, as it has previously been described to activate *Pax2* (Watanabe *et al.*, 2007). Curiously, transfection with pBI-Meso1 resulted in general downregulation with occasional upregulation of *Pax3*, more closely resembling the effects of pBI-Pax3 on *Pax7* than pBI-Meso1 on *Pax2*.

It is clear in each case that transfected cell clusters are heterogeneous in terms of their expression of tissue identity markers. The mechanisms underlying this heterogeneity are still obscure, and may represent stochastic activation of counter-inhibitory gene networks, or alterations to local signalling events.

## Adhesion

The formation of cell clusters which, in some cases, separate themselves from the surrounding tissue is one of the most striking features of pBI-Pax3/7 electroporation. This is supported by our observation that the cell adhesion molecule NCAM is increased in transfected tissue. This is in stark contrast to *in vitro* studies, where *Pax3* does not directly regulate *NCAM* expression (Chalepakis *et al.*, 1994). It will be interesting to investigate whether *Pax3* regulates NCAM *in vivo*, possibly modulated by other factors.

While it is tempting to suggest that the regulation of *NCAM* by pBI-Pax3/7 is significant for the process of somitogenesis, the role of NCAM in the paraxial mesoderm has not been clearly defined. Although it is expressed in the PSM, mice lacking *NCAM* develop normally, suggesting near complete redundancy in the paraxial mesoderm (Cremer *et al.*, 1994). While we have shown that ectopic *NCAM* is sufficient to drive a cell sorting process, producing clusters of transfected cells, the function of NCAM in the paraxial mesoderm is not clear. Furthermore, the regulation of *NCAM* by other factors such as *Pax2* suggests that the regulation of *NCAM* in the paraxial mesoderm may be redundant between multiple Pax genes.

Interestingly, knockouts of the sialyltransferases required for polysialylation of NCAM produce a more severe phenotype, although this appears to be broadly restricted to the patterning of the nervous system (Weinhold *et al.*, 2005). The interesting role of polysialic acid on NCAM function, combined with reports

that PSA-NCAM is affected in *Pax3* mutants prompted us to examine whether polysialylation was being affected in by transfection with pBI-Pax3/7. While our preliminary results suggest that PSA-NCAM is not upregulated by pBI-Pax3/7, we did observe an interesting spatial distribution of PSA-NCAM which has not previously been described (see Appendix 2).

## Cytoskeleton and Epithelialisation

Because of the finding that ectopic *Pax3* is capable of driving a mesenchymal-to-epithelial transition in cell cultures (Wiggan *et al.*, 2002), it's expression in the PSM immediately prior to segmentation (Schubert *et al.*, 2001), and it's maintenance in the epithelial dermomyotome (Dietrich and Gruss, 1995), it is tempting to speculate about a role for *Pax3* in promoting epithelialisation. Alternatively, recent research has suggested that epithelialisation begins in the medial PSM, where *Pax3* is at it's lowest (Martins *et al.*, 2009). We wanted to examine the role of *Pax3* in epithelialisation of the rostral PSM. When looking at the cytoskeleton, we found that F-actin was strongly upregulated upon transfection (see Appendix 4), while levels of β-tubulin were not affected (not shown). However, we were not able to image these tissues with enough resolution to uncover more subtle changes in distribution.

Cadherins are important components of the adherens junction, which are localised to the apicolateral surfaces of cells in epithelia. The localisation of N-cadherin and F-actin to the apical (internal) surface of the somite is an important component of somitogenesis, marking the formation of an epithelial

somite (Martins *et al.*, 2009). N-cadherins are upregulated in the rostral PSM (Duband *et al.*, 1987), and subjected to rapid reorganisation when epithelialisation occurs, potentially under the regulation of Rho-family GTP-ases (Nakaya *et al.*, 2004). We could not detect upregulation of N-cadherin in pBI-Pax3/7 expressing clusters, or reorganisation of endogenous N-cadherin (see Appendix 4). Moreover, *Pax3* prevented the reorganisation of N-cadherin in normal segmentation. In contrast, transfection with *Paraxis*, a factor associated with the formation of the somitic epithelium (Burgess *et al.*, 1996) produced clusters with a rosette-like structure, with both N-cadherin and F-actin localised to the interior surface, reminiscent of ectopic somites.

Interestingly, while pBI-Pax3 does not appear to promote epithelial identity, it is sufficient to maintain it in the sclerotome. The sclerotome loses it's epithelial character, including N-cadherin expression, however pBI-Pax3 transfected cells in the maturing paraxial mesoderm retained an epithelial organisation and N-cadherin, presumably reflecting a dermomyotomal identity over sclerotome.

## Fissure Formation and Segmentation

We were also interested to note that in many cases, pBI-Pax3/7 transfected cell clusters became detatched from the non-transfected PSM tissue. It should be noted that these fissures were observed after cryosectioning, and may be exacerbated by the fixation, cryopreservation and sectioning processes, and may not represent a physical separation between tissues in

live embryos. However, it does imply that transfected tissue was less well attached to the surrounding tissue. Interestingly wherever these gaps appear, a fibronectin matrix is assembled. Fibronectin is an important component of the extracellular matrix, which has an important role in maintaining separation between tissues in the developing embryo.

We have identified three potential mechanisms for the formation of intercellular fissures at the borders of transfected tissue: differential adhesion, assembly of a new extracellular matrix, and co-option of signalling pathways involved in formation of an intersomitic gap (fig. 4.1).

Differential cell adhesion is known to facillitate cell sorting and the production of clear boundaries within tissues, either through a process of heterologous expression of different adhesion molecules (Cooke *et al.*, 2005), or the expression of a single adhesion molecule at different concentrations (Foty and Steinberg, 2005). Cell sorting certainly occurs within the pBI-Pax3/7 transfected PSM, as evidenced by the formation of cell clusters. Since all known adhesion molecules are expressed both in transfected and untransfected domains, with at least one (NCAM) being strongly upregulated in transfected cells, the Differential Adhesion Hypothesis (DAH) proposed by formation, in which transfected tissue loses its connection with adjacent untransfected tissue. According to the DAH, this is can only be achieved

using simple thermodynamics if there is no cross-adhesion between the two populations (Foty and Steinberg, 2004). Consistent with this model, transfection with pBI-NCAM induces a level of clustering, but does not appear to produce fissures.

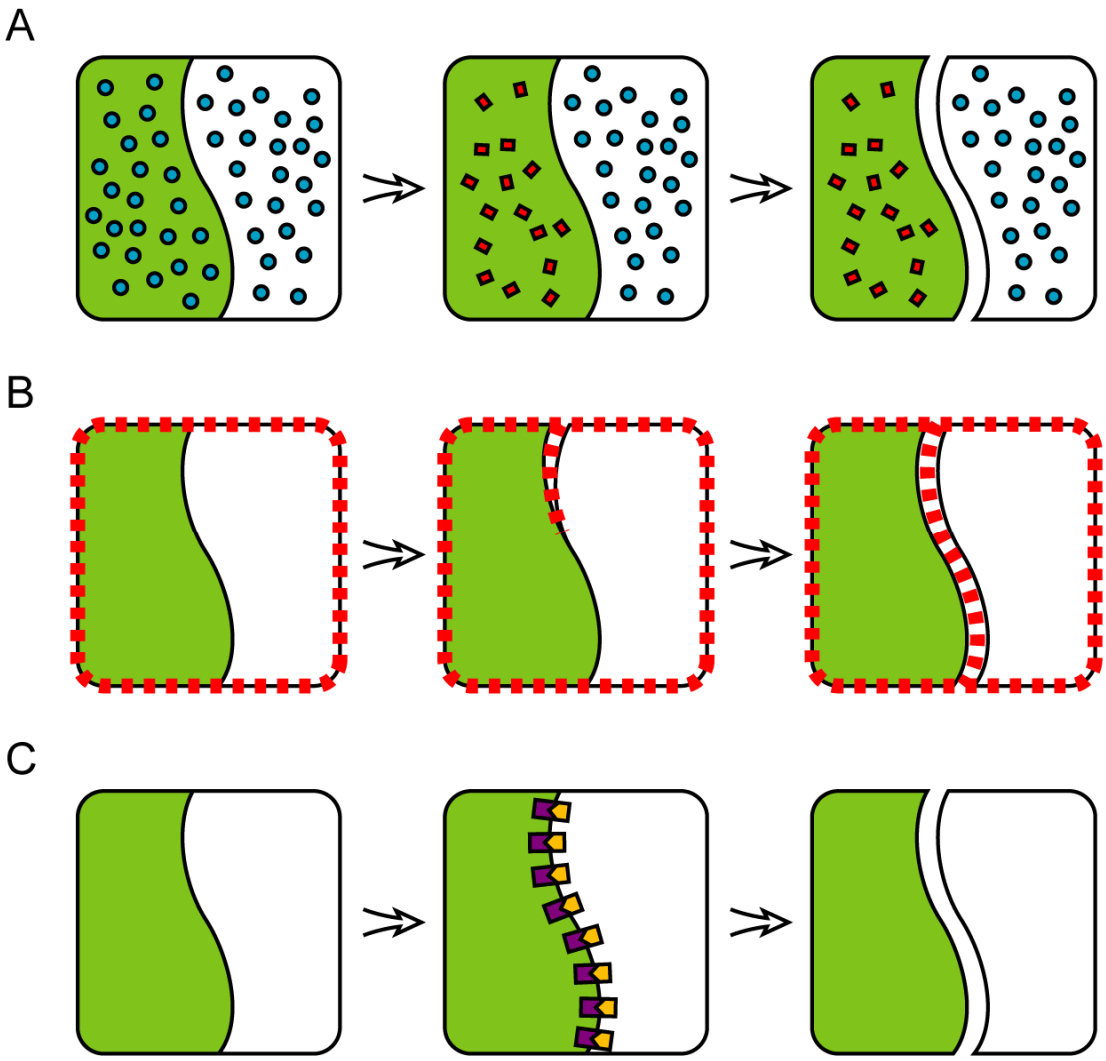


**Figure 4.1: Models for Fissure Formation**

A: Differential adhesion. Transfected tissue expresses a different complement of CAMs and detatches from the surrounding tissue. B: Upregulation of extracellular matrix. Ectopic matrix is produced at the edges transfected tissue, forcing it to detatch. C: Co-option of signalling pathways. Transfection transiently activates signalling events which produce an ectopic intersomitic gap.

Fibronectin monomers are assembled into a matrix in the extracellular space through an active process mediated by integrins. While it is formally possible that fibronectin may be assembled within a tissue, and indeed short fibrils are described within the PSM (Duband *et al.*, 1987), assembly of a fibronectin matrix is usually restricted to the surface of tissues (Julich *et al.*, 2009). Since the fibronectin matrix effectively acts as a physical barrier preventing cellular intermixing between tissues, it is possible that fissures may be formed as a result of ectopic matrix assembly driving a wedge between the two domains. It has recently been suggested that trans-interactions between integrins on adjacent cells inhibits fibronectin fibrillogenesis in zebrafish, and a border between high and low integrin expression domains will induce assembly of a fibronectin matrix (Julich *et al.*, 2009). They also noted that surgical separation of PSM tissue was sufficient to induce fibrillogenesis in some cases. This raises two potential scenarios to explain the fibronectin expression pattern. In the first scenario, integrin expression is altered between transfected and non-transfected tissue, actively driving fibrillogenesis and subsequently fissure formation. Alternatively, pBI-Pax3/7 drives fissure formation through unknown means, releasing trans-inhibition of integrins, and causing fibrillogenesis as a consequence. Further investigation will be necessary to definitively establish casuality in this system.

While transfection with pBI-Pax3/7 evidently does not produce a polarised somitic epithelium, the formation of a fissure between transfected and

untransfected tissue might be considered analogous to the intersomitic gap. It should also be noted that transfected tissue does not segment properly.

According to the current model of segmentation, MesP/Meso forms a tightly apposed expression domain with activated Notch signalling (Oginuma *et al.*, 2008). This arrangement is thought to produce a tightly apposed domain of *EphA4* in the anterior of S-1 and *EphrinB2* in the posterior of S0, which is sufficient to drive the formation of an intersomitic gap (Watanabe *et al.*, 2009).

It should first be noted that pBI-Pax3/7 transfected cells downregulate *Tbx6*, which is required for *MesP2* expression in mouse (Oginuma *et al.*, 2008). Indeed, pBI-Pax3/7 transfected cells do not express *Meso1*, although since *Meso1* is transiently expressed, it is formally possible that it is transiently expressed in transfected tissue and downregulated over the course of the culture period. It is interesting to note that transfection of pBI-Meso1 into the PSM does not appear to result in fissure formation in the same way as pBI-Pax3/7, although in some cases, a fibronectin matrix can be detected at the borders of *Meso1* transfected tissue.

Transfection with pBI-Pax3/7 may also modulate Notch signalling. We have not investigated the effect of pBI-Pax3/7 on Notch signalling components such as the ligand *Delta1* or the downstream target *Lfng*, however it seems likely that the loss of *Tbx6* will eventually result in loss of it's downstream target *Delta1*, interrupting Notch signalling in transfected tissue (Hofmann *et al.*,

2004, White and Chapman, 2005). If so, it may be interesting to investigate whether local disruption of Notch signalling, for example using the domninant negative *Suppressor of Hairless* construct, recapitulates part of the pBI-Pax3/7 fissure phenotype.

It is possible that the boundary created by electroporated clusters may alter or activate signalling events which drive gap formation, such as EphA4-EphrinB2 signalling. We did not detect expression of either *EphA4* or *EphrinB2* in or around transfected clusters (see Appendix 5), although like *Meso1*, their endogenous expression pattern is transient and may be downregulated within the culture period. Careful time-course analysis, or even live imaging of the behaviour of transfected tissue at a cellular level may resolve some of these issues.

Investigating the effect of pBI-Pax3/7 using the intersomitic gap grafting assay (Sato *et al.*, 2002, Watanabe *et al.*, 2009) may be informative, however it should be noted that the factors identified using this technique are dynamically regulated components of the segmentation process which were tonically expressed during the assay. If *Pax3/7* transiently induces pathways involved in intersomitic gap formation, they may not be expressed long enough to register in the grafting assay.

# Loss of Function

The effect of a loss of function of *Pax3*, and to a lesser extent *Pax7*, have been extensively studied in mice using a wide array of knockout and hypomorphic mutants, however the majority of these have concentrated on the effects of *Pax3* in the myogenic lineage rather than morphogenetic effects at the level of somite formation. A mild dysregulation of segment formation has been observed (Schubert *et al.*, 2001), but a clear analysis of the function of *Pax3* is confounded by extensive redundancy with other genes involved in segmentation, particularily Pax7 (Relaix *et al.*, 2004). Mice mutant for both *Pax3* and *Pax7* have extensive defects in the dermomyotome, but an analysis of effects on segmentation have not been investigated. It is also important to note that the expression pattern of *Pax3* in the chicken embryo is very different from the expression pattern in the mouse.

Loss of function experiments are likely to be very important in defining the function of *Pax3* in the PSM. We have investigated the possibility of using small hairpin RNA constructs to knockdown expression of *Pax3/7* (see Appendix 6). While this worked in the neural tube, we had technical issues effectively transfecting the PSM. We also investigated the possibility of using morpholino's, but were unable to induce any knockdown effect. If the technical limitations can be overcome, these techniques may provide valuable insights into the role of Pax3 and Pax7 in segmentation.

# Conclusion

*Pax3* and *Pax7* are important regulators of morphogenesis in the developing embryo. We have examined the expression of *Pax3/7* in the chick PSM, and performed a preliminary analysis of signalling pathways which may be regulating these factors. Additionally, we have developed a method of transfecting the chick PSM with ectopic *Pax3/7*, and have examined its effect on tissue identity, cell adhesion and extracellular matrix formation.

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# Appendix 1: Regulation of *Pax3*

*Pax3* shows an interesting expression pattern in the rostral PSM which may be consistent with dynamic regulation downstream of the segmentation clock (fig. 3.1). We have performed a preliminary investigation into the mechanism by which *Pax3* is regulated. Firstly, we have investigated whether expression of *Pax3* mRNA may vary in time with the segmentation clock. Secondly, we have considered the role of specific signalling pathways in the regulation of *Pax3*. In both cases, we have performed specific experiments whose results did not achieve the required standards for inclusion in this results section of this thesis. For completeness, they are included here with the caveat that they represent very preliminary data, and should be interpreted with caution.

## *Pax3* and the Segmentation Clock

We have used a fix and culture technique to try and evaluate how the expression pattern of *Pax3* changes throughout the segmentation clock cycle, however the results do not appear to reflect the pattern seen in complete embryos (fig. 3.2). We were concerned that the culture system required for the fix and culture technique might be affecting the expression pattern of *Pax3* in these explants.

As an alternative, we decided to compare expression of *Pax3* to *Lfng*, a modulator and downstream target of the Notch pathway which exhibits characteristic changes in expression pattern at different phases of the

segmentation clock cycle (fig A.1a). Subjecting two identical and uncultured PSM explants from the same embryo to *in situ* hybridisation for either *Pax3* or *Lfng* allows us to identify the presence or absence of rostral bands during a known phase of the segmentation clock cycle. Further, this should eliminate any complications that might arise as a result of subjecting the explants to culture conditions.

Frustratingly, we were still unable to achieve reliable results performing *in situ* hybridisation on uncultured explants, with only a minority exhibiting bands in the rostral PSM (fig A1b,c, n=2/12). However, it was possible to identify two explant pairs which exhibited the same phase of *Lfng* expression but different patterns of *Pax3* in the rostral PSM. If correct, this result would suggest that the variations of *Pax3* identified in this system do not correspond to phases of the segmentation clock cycle, however it should be noted that more examples must be found before drawing any conclusions.

## *Pax3* and *Snail2*

*Snail2* is gene which cycles in the PSM in time with the segmentation clock, but has a very different expression profile to *Lfng*. In the rostral PSM, *Snail2* exhibits a band of expression at around the same level as the caudal-most of the rostral bands of *Pax3* (fig. A1d,e). We compared uncultured half embryo pairs for expression of *Snail2* and *Pax3*. As noted above, for technical reasons this approach is not suitable for a systematic comparison of *Snail2*

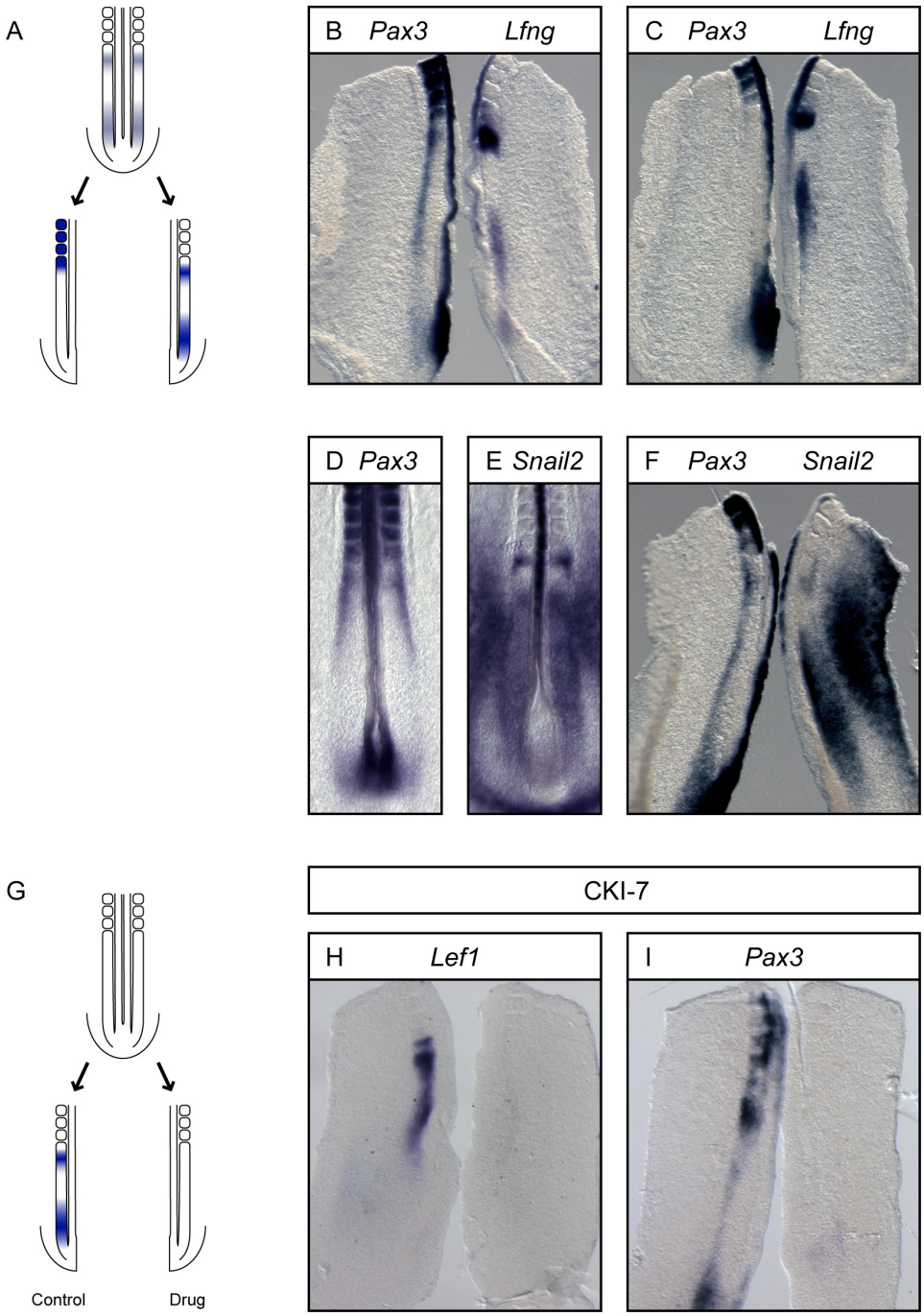


**Figure A.1: Regulation of *Pax3***

A-C: comparison of *Pax3* during segmentation cycle using half-embryo technique. Both explants are fixed together, allowing analysis of *Pax3* expression at known phases of the segmentation clock cycle. Phase is established by comparing with known cycling gene *Lfng* (A). Rostral bands of *Pax3* were only observed in a minority of embryos (B, n=2/12) while most did not exhibit bands in the rostral PSM (C,

**cont. overleaf**

and *Pax3* throughout the segmentation cycle, however we were able to identify a minority (n=2/12) of explant pairs which exhibited *Pax3* expression in the rostral PSM (fig. A1f). Interestingly, in these cases, *Pax3* was expressed at the same level as *Snail2* expression domain.

This result may suggest that common signalling events regulate expression of *Pax3* and *Snail2*. Alternatively, given the potential repressive role of *Snail2* on genes such as *Pax3*, *Snail2* expression at this level may produce the band of low *Pax3* expression in the rostral PSM. However, because of the low number of clearly stained embryos, it is not possible to draw firm conclusions from this data.

## Wnt Signalling and *Pax3*

We also considered the effect of cell signalling pathways on *Pax3* expression. Interfering with Notch signalling disrupts the segmentation clock and prevents formation of clear segment boundaries, but does not prevent the upregulation of *Pax3* in the rostral PSM (fig. 3.2). Another important signalling pathway in segmentation is the Wnt pathway. Wnt is known to be important in the

**Figure A.1: Regulation of *Pax3* cont.**

n=10/12). The presence or absence of *Pax3* rostral bands did not appear to vary with segmentation clock phase as assayed by *Lfng*. D-F: comparison of *Pax3* to the mesodermal factor *Snail2*. During part of its cycle, *Snail2* exhibits rostral bands (D, n=2/4) which may be related to the pattern of *Pax3*. In uncultured half embryo explants, bands of *Pax3* and *Snail2* appear at the same level in the PSM (F, n=2/12). G-I: effect of casein kinase inhibitor CKI-7 on Wnt target *Lef1* (H, n=2) and *Pax3* (I, n=1) in half embryo culture over 3 hours.

regulation of *Pax3* later in segmental patterning, so it is a strong candidate for affecting *Pax3* in the rostral PSM.

Wnt signalling can be disrupted in the paraxial mesoderm using the small molecule inhibitor CKI-7 (Chijiwa *et al.*, 1989). For technical reasons it is not possible to use this inhibitor in whole-embryo culture systems (such as EC culture), so we used a half embryo culture system. We did find a clear downregulation of *Pax3* in treated embryos (fig. A.1i, n=1), although at extremely low numbers. Combined with the technical problems we have detailed on detecting *Pax3* in half embryos, this data should be regarded with extreme caution.

In summary, we were not able to perform a thorough investigation of the dynamic regulation of *Pax3* in the rostral PSM due to a technical problem with *in situ* hybridisation on PSM explants. Rostral expression of *Pax3* appears to be spatially correlated with rostral bands of *Snail2* expression. Finally, Wnt signalling appears to be required for *Pax3* expression in the PSM.

# Appendix 2: Exogenous *NCAM* and Polysialylation of NCAM in response to *Pax3*

We have developed a technique which allows us to transfect the PSM of chicken embryos with a transgene without interfering with primitive streak or tailbud morphogenesis (fig. 3.3 and 3.4). When we transfect the PSM with a construct containing the *Pax3/7* genes (pBI-Pax3 and pBI-Pax7), we observe a range of effects on cell and tissue morphology, including cell clustering and disruption of the segmentation process (fig. 3.4). We also observed an upregulation of the cell adhesion molecule NCAM (fig. 3.7). Since an increase in adhesion may underlie parts of the phenotype of pBI-Pax3/7 transfected embryos, we were interested in investigating the extent to which exogenous NCAM can recapitulate this phenotype.

NCAM has three major isoforms produced by truncation of the C-terminal intracellular domain by alternative splicing of the mRNA transcript. The shortest isoform is exclusively associated with the nervous system (Hemperly *et al.*, 1986), while the longest is predominantly expressed later in development (Murray *et al.*, 1986). Consistent with this, when we cloned *NCAM* from a HH12 whole chick embryo cDNA library, we produced only clones containing the 140 kDa isoform of NCAM (NCAM-140, fig. A.2a,b).

We subcloned the full length *NCAM* cDNA into the doxicycline-inducible plasmid and transfected it into the PSM. The identity of the cloned gene was confirmed by sequencing and immunohistology. In whole mount it appeared to have a similar clustering effect to pBI-Pax3/7, although cluster sizes were smaller (fig. A.2c, fig. 3.5a). In one case, we saw multiple fused somites in a single embryo (n=1/6, fig. A.2d, arrowheads), but otherwise pBI-NCAM-transfected cells were correctly incorporated into the somites, suggesting that NCAM upregulation alone does not explain the pBI-Pax3 transfected phenotype. Similarily, we were unable to detect fissures between transfected and non-transfected tissue (fig. A.2e, fig. 3.5b).

This suggests that while increases in NCAM may underlie cell clustering in pBI-Pax3/7-transfected embryos, it does not reproduce the other effects, which may be a result of changes in cell identity in transfected cells.

The potential role of NCAM is further complicated by its state of glycosylation. NCAM is subject to a unique sialic acid modification, producing a polysialylated form (PSA-NCAM) which has differential effects on cell adhesion and motility (fig. A.2f). This modification can be removed *in vitro* by treating with Protein N-glycosidase F (PNGase F, fig. A.2g). There have been varying reports of the effect of Pax3 on the levels of PSA-NCAM (Neale and Trasler, 1994; Glogarova and Buckiova, 2004) and a sialyltransferase responsible for NCAM polysialylation is transcriptionally regulated by *Pax3* (Mayanil *et al.*, 2001). For this reason, we decided to investigate PSA-NCAM.

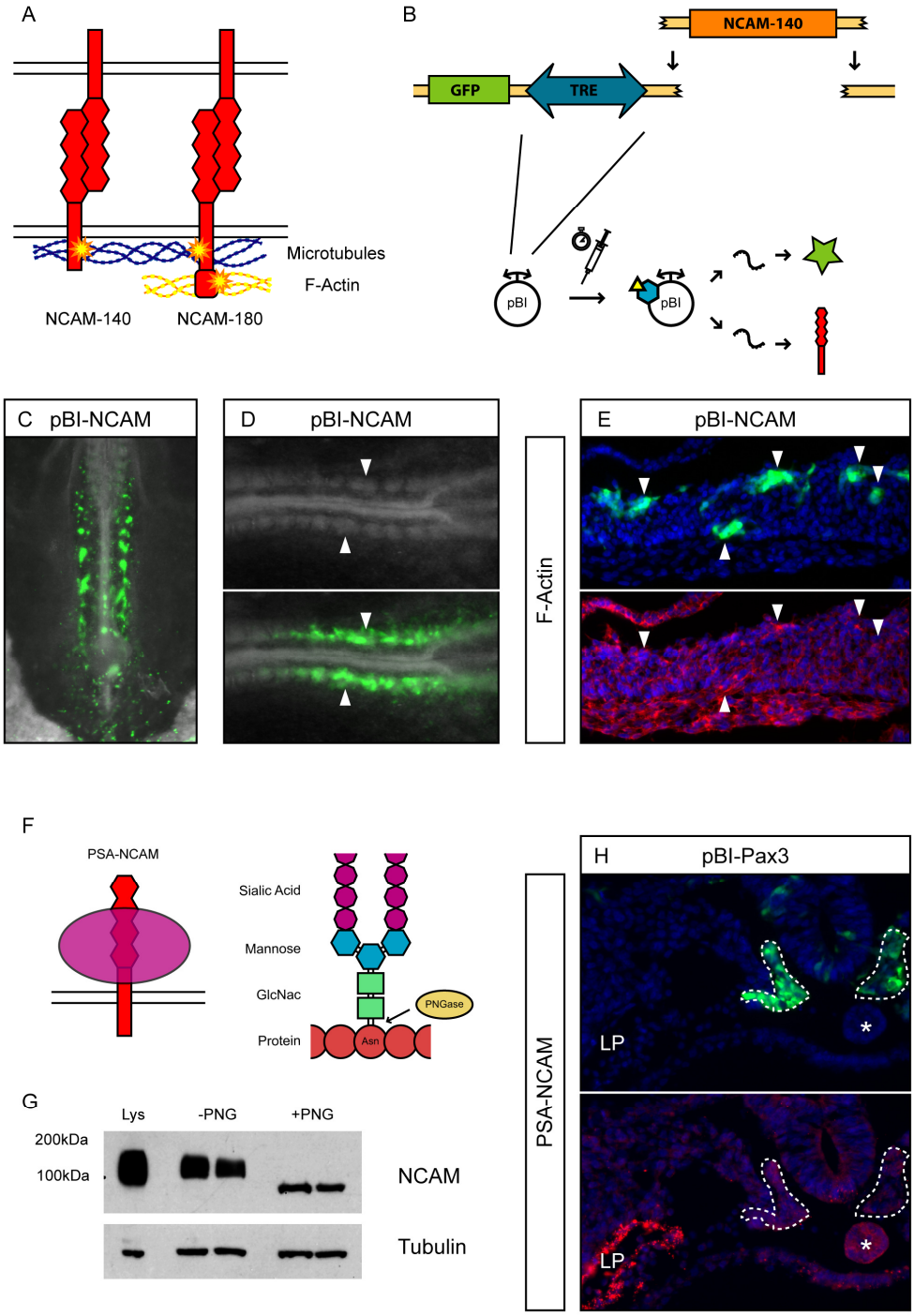


**Figure A.2: Transfection of *NCAM* and regulation of NCAM glycosylation by *Pax3***

A-E: cloning and transfection of pBI-NCAM. The 140kD variant of *NCAM* was cloned and inserted into the doxicycline inducible plasmid (A-B). Transfection with NCAM recapitulates part of the Pax3/7 phenotype (C-E). Cells transfected with pBI-NCAM appear to form cell clusters (C, n=6) and occasionally cause fused somites (D, n=1/6),

**cont. overleaf**

Our first concern was that our immunohistological data may be affected by the polysialylation of NCAM. To check that our NCAM antibody worked equally well on both PSA-NCAM and non-glycosylated NCAM, we treated HH12 whole chick lysates with PNGase F to remove the polysialic acid residues and performed a Western blot. Bands are clearly visible in both treated and control incubations, demonstrating that the NCAM antibody binds NCAM and PSA-NCAM (fig. A.2g). However, we cannot rule out the possibility that the antibody binds to NCAM and PSA-NCAM with different affinities.

To investigate whether the NCAM produced by pBI-Pax3 transfection is polysialylated, we used a PSA-NCAM specific antibody. PSA-NCAM has not been well characterised in the early chick embryo, but we detected strong, punctate staining in the lumen of the lateral plate, in the notochord and at the floor plate (fig. A.2h, asterisk and LP, and data not shown). We detected only very faint staining in pBI-Pax3 transfected cells, suggesting that the NCAM variant induced by *Pax3/7* overexpression is not polysialylated.

**Figure A.2: Transfection of *NCAM* and regulation of NCAM glycosylation by *Pax3* cont**

however NCAM-transfected cells do not separate from the surrounding PSM (E, arrowheads, n=3). F-H: Polysialylation of NCAM is not affected by Pax3. In some contexts, NCAM may be subject to post-translational modification by the addition of polysialic acid. This modification stems from an Asparagine-GlcNac residue which can be removed using a PNGase enzyme (F). Comparison of PNGase digested samples (+PNG) with non-enzyme (-PNG) and lysate controls by Western blot shows that embryonic NCAM is subject to glycosylation, and this does not affect the affinity of our anti-NCAM polyclonal antibody (G, n=1). Analysis of embryos using a PSA-NCAM specific antibody reveals strong punctate staining in the lateral plate (H, LP) and even staining throughout the neural tube and notochord (asterisk). Clear upregulation by transfection with pBI-Pax3 was not observed. (H, n=1).

From these results it would appear that Pax3 does not have a clear effect on the polysialylation of NCAM in the PSM, and the observed affects are due to the increase in NCAM expression rather than a change in its level of polysialylation. However, it should be noted that insufficient numbers have been analysed to draw clear conclusions.

# Appendix 3: N-Cadherin and the Cytoskeleton

We have developed a technique which allows us to transfect the paraxial mesoderm of chicken embryos with a transgene without interfering with primitive streak or tailbud morphogenesis (fig. 3.3 and 3.4). When we transfect with a construct containing the *Pax3/7* genes (pBI-Pax3 and pBI-Pax7), we observe a range of effects on cell and tissue morphology, including cell clustering and disruption of the segmentation process (fig. 3.4). Because *Pax3* has been implicated in epithelialisation (Dietrich *et al.*, 1998, Wiggan *et al.*, 2002) we wanted to see whether Pax3 affected epithelialisation in our transfection assay. Due to the low numbers of samples, these results represent a preliminary investigation, and all conclusions should be considered provisional.

## N-cadherin

N-cadherin is an important cell adhesion molecule in the maturation of paraxial mesoderm. In epithelia, cadherins are typically associated with the zonula adherens at the apical border of epithelial cells. N-cadherin is generally associated with mesenchymal cell adhesion, and is expressed throughout the mesenchymal PSM (Duband *et al.*, 1987). However, N-cadherin is also apically localised in the atypical epithelia of the neural tube and somites. N-cadherin is lost completely when sclerotome undergoes an EMT. In contrast to NCAM, N-cadherin is not upregulated by pBI-Meso1 or

pBI-Pax2 (Watanabe *et al.*, 2007). Consistent with these observations, we could not reliably detect an upregulation of N-cadherin in pBI-GFP (n=1), pBI-Pax3 (n=6) or pBI-Pax7 (n=1) transfected cells in the rostral PSM (fig A.3a, b, d). Interestingly, in mature somites where the sclerotome had undergone EMT and downregulated N-cadherin, the protein was still detected in pBI-Pax3 transfected tissue (n=2, fig. A.3c). It is unclear whether this represents

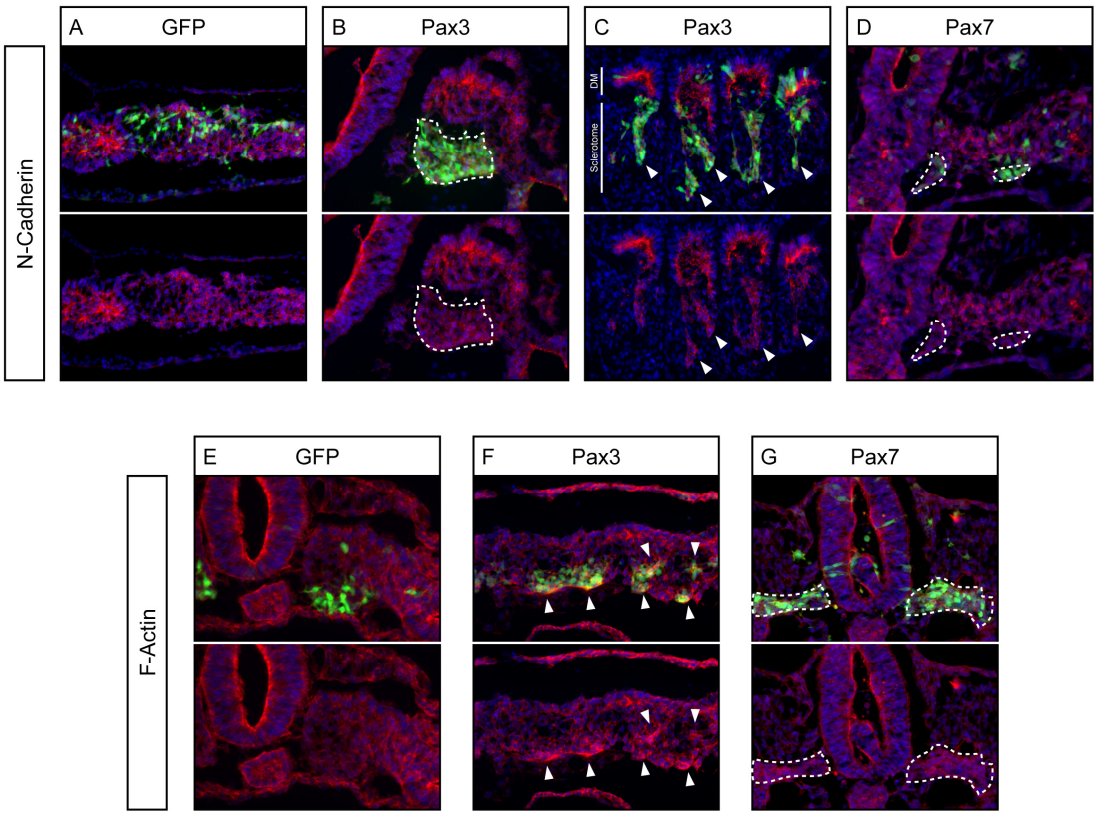


**Figure A.3: Effect of *Pax3/7* on N-Cadherin, and F-Actin**

A-D: Transfection with pBI-GFP (A, shown in longitudinal section, n=1), pBI-Pax3 (B, n=6) or pBI-Pax7 (D, n=1) does not affect N-cadherin in the PSM. pBI-Pax3 also prevents N-cadherin from becoming polarised (B). N-cadherin is maintained in pBI-Pax3 transfected cells in the sclerotome (C, shown in longitudinal section, n=2). E-G: Transfection with pBI-GFP has no effect (E, n=1), but pBI-Pax3 (F, shown in longitudinal section, n=2) and pBI-Pax7 (G, n=1) both induce upregulation of F-actin in the PSM.

a direct interaction between *Pax3* and N-cadherin or is a consequence of failure to adopt sclerotomal fate. However, despite maintaining N-cadherin at comparable levels to the adjacent PSM and somites, pBI-Pax3 transfected cells failed to organise N-cadherin into an apical domain typical of a polarised epithelium (fig. A.3b).

In summary, *Pax3/7* appears to have a role in promoting cell adhesion via NCAM, but does not have a clear effect on N-cadherin. Since establishment of a polarised epithelium involves the apical localisation of N-cadherin, this suggests that *Pax3/7* does not produce epithelialisation in the paraxial mesoderm. However, it is possible that *Pax3* may promote other factors required for MET, such as components of the cytoskeleton and extracellular matrix.

## The Cytoskeleton

As PSM undergoes MET, the cytoskeleton undergoes a dramatic reorganisation, with characteristic changes in F-actin and microtubule distribution. The actin cytoskeleton may mediate some of the effects of eph-ephrin signalling, and is also localised to the apical surface of the somitic epithelium as the adherens junction forms. Even after MET, cells in the somitic epithelium are unusually dynamic (Martins *et al.*, 2009). It has also been shown that cell lines transfected with *Pax3* reorganise actin and tubulin *in vitro* (Wiggan *et al.*, 2002).

Cells transfected with pBI-Pax3 (n=2) and pBI-Pax7 (n=1) exhibited increased staining for F-actin (fig. A.3f,g). Since F-actin represents only a subset of the actin protein within the cell, it is not possible to assess whether this represents an upregulation of total actin, or an increase in actin polymerisation. However, as with N-cadherin, the actin cytoskeleton did not become polarised as it does in a mature somitic epithelium. Conversely, no effect was distinguishable on β-tubulin (not shown), however we were unable to achieve sufficient resolution to examine microtubule distribution.

In summary, transfection of the chicken paraxial mesoderm with pBI-Pax3/7 does not appear to induce epithelialisation in unsegmented tissue as assayed by upregulation or organisation of N-cadherin or β-tubulin. Changes in the actin cytoskeleton were detected, but these did not appear to be related to MET. However it should be remembered that these results are based on a very small sample size, and should be considered provisional.

# Appendix 4: Transfection of *Myf5*

The initial patterning of the musculature during myogenesis depends on a complex network of transcription factors acting on the paraxial mesoderm. Among the earliest expressed genes in this network is the myogenic factor *Myf5*. It has been shown that Pax3 regulates Myf5 (Maroto *et al.*, 1997), and that the Myf5 promoter contains binding sites for Pax3 (Bajard *et al.*, 2006). We have successfully shown that exogenous *Pax3/7* results in an upregulation of *Myf5* in a subset of transfected cells (fig. 3.6f-j). A similar effect is observed on *Pax7* when transfected with pBI-Pax3 (fig 3.10a). We were interested in whether exogenous *Myf5* could produce a reciprocal effect on *Pax3*.

Unlike *Pax3/7*, *Myf5* can be transfected via the primitive streak using the expression vector pCIG (fig. 3.3d) without disrupting gastrulation (fig. A.4a). Surprisingly, transfection with pCIG-Myf5 also causes upregulation of NCAM (n=1), but does not disrupt either gastrulation or segmentation (fig. A.4c, n=1). pCIG-Myf5 did not appear to have an effect on PSM identity (as assayed by *Tbx6* expression, n=5, fig. A.4b), although this remains to be confirmed by cryosection.

In order to investigate whether pCIG-Myf5 may be acting via *Pax3*, we assayed pCIG-Myf5 transfected embryos for expression of Pax3 protein, and found that, similar to the effect of pBI-Pax3 on *Pax7*, isolated cells in pCIG-

Myf5 transfected embryos showed upregulation of Pax3 (n=1, fig. A.4d, arrowheads). Conversely, some clusters of transfected cells did not show

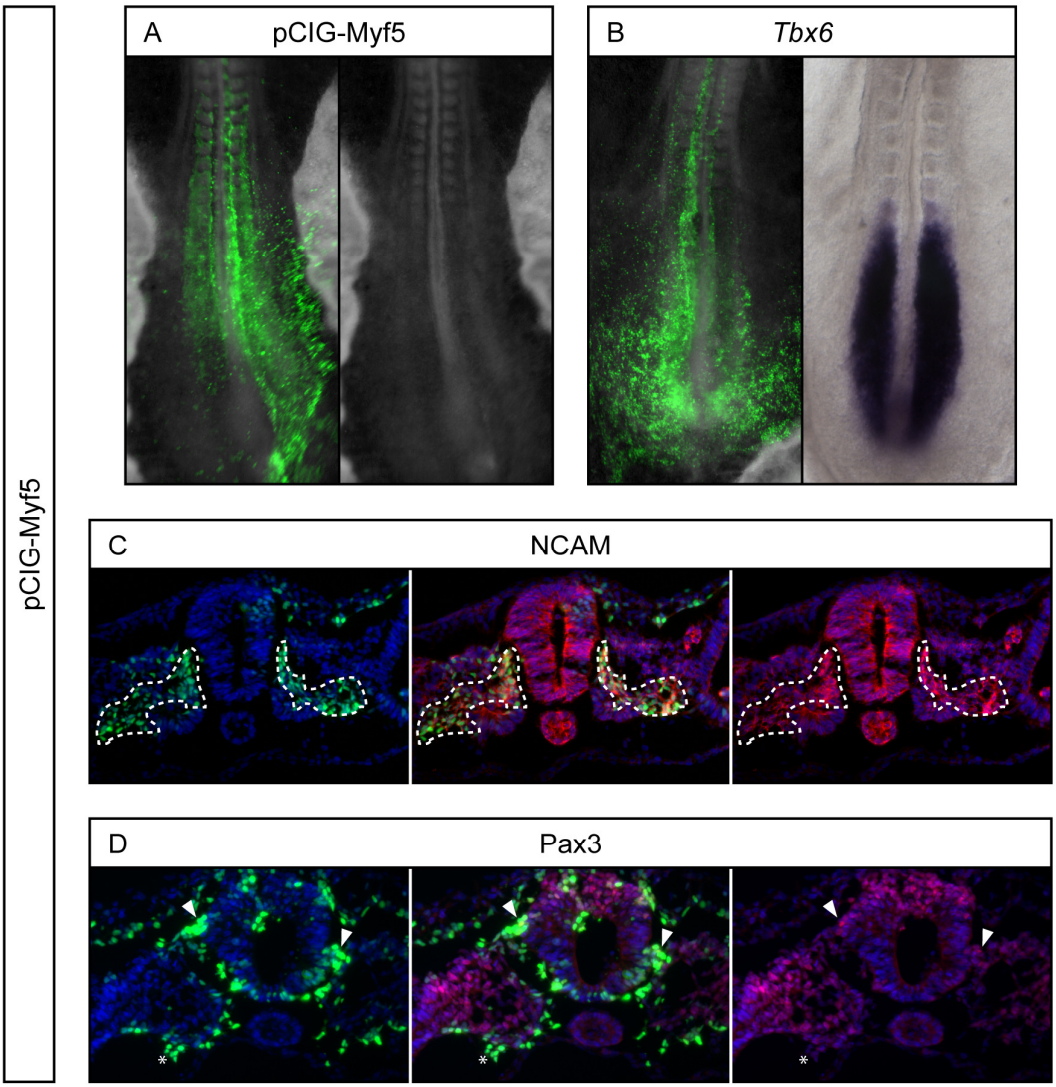


**Figure A.4: Transfection of *Myf5***

pCIG-Myf5 does not affect morphogenesis of the PSM (A, n=7) or *Tbx6* expression (B, n=5). pCIG-Myf5 also upregulates NCAM (E, n=1), and may upregulate Pax3 in a small number of cells (F, n=1).

increased Pax3 (n=1, fig. A.4d, asterisk). However, due to the low number of embryos considered, these results should be considered preliminary.

In summary, *Myf5* may represent part of a network of myogenic transcription factors active in the early PSM. The interactions of these factors upon each other is likely to be complex, and warrants further investigation.

# Appendix 5: Eph-Ephrin Signalling

Transfecting the PSM with ectopic *Pax3/7* causes an interesting morphological effect on cells in this tissue. Transfected cells form clusters which in many cases are separated from the surrounding tissue by fissures (fig. 3.5). Changes in adhesion are sufficient to explain the formation of cell clusters (Foty and Steinberg, 2005) but not the formation of fissures. The possibility that pBI-Pax3/7 may be co-opting part of the segmentation machinery to produce fissures is an intriguing one.

Segmental borders are defined by the apposition of *MesP* and activated Notch signalling (Morimoto *et al.*, 2005). *EphA4* is thought to be downstream of *MesP* (Nakajima *et al.*, 2006, Watanabe *et al.*, 2009), while *EphrinB2* is downstream of Notch (Grego-Bessa *et al.*, 2007). We examined whether ectopic *Pax3* had an effect on expression of *EphA4* (fig. A.5c, n=6) or *EphrinB2* (fig A.5f, n=1) in the anterior PSM, but could not detect any effect.

It should be remembered that *EphA4*, like *MesP*, is only expressed transiently in the PSM. If ectopic pBI-Pax3/7 is having a positive effect on these factors, the time course of the experiment may prevent us from detecting it. It should also be noted that the number of *EphrinB2* samples is too low to draw clear conclusions.

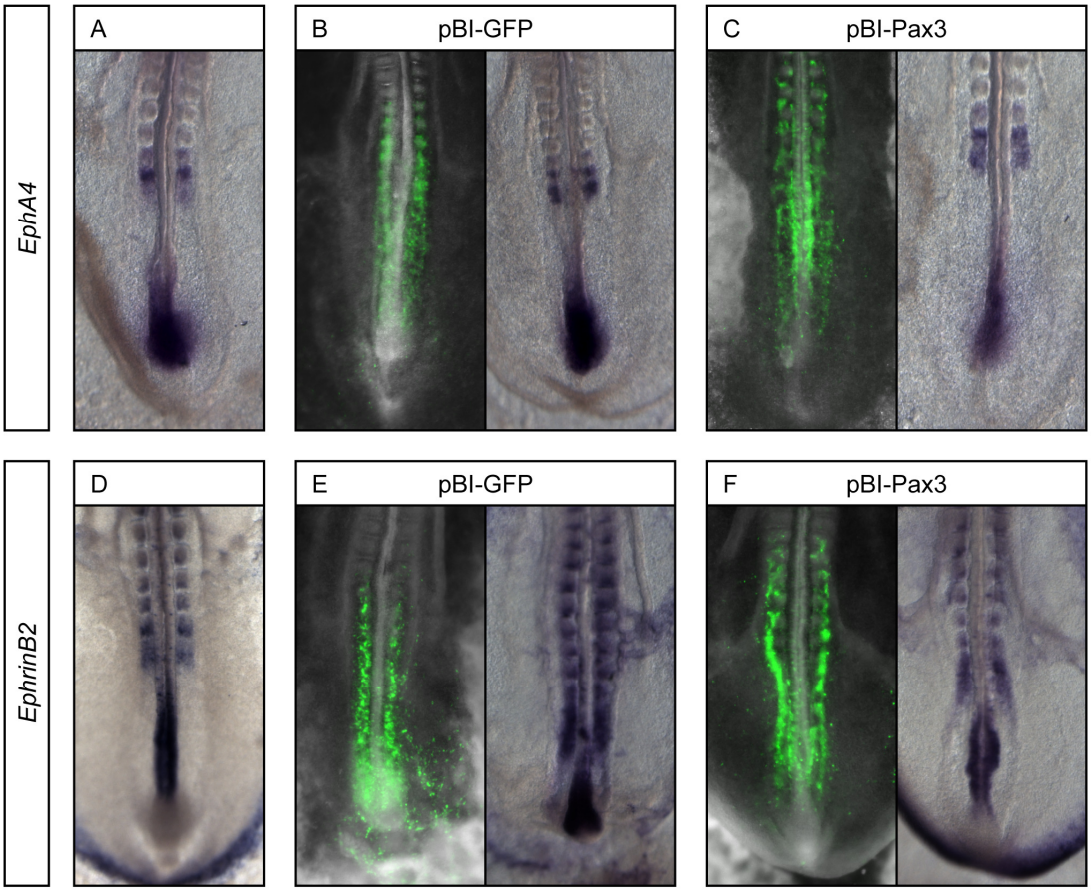


**Figure A.5: Effect of pBI-Pax3 Transfection of Eph-Ephrin Signalling**

A-C: Expression of *EphA4* mRNA. *EphA4* is not affected by either pBI-GFP (B, n=2) or pBI-Pax3 (C, n=6). D-E: Expression of *EphrinB2* mRNA, *EphrinB2* is not affected by either pBI-GFP (E, n=1) or pBI-Pax3 (F, n=1).

# Appendix 6: Genetic Disruption of Pax3/7

We have developed a transfection assay which allows us to cause a gain of function of *Pax3/7* in the chick PSM. In order to properly understand the role of *Pax3* and *Pax7* in development, it will be necessary to consider the effect of disrupting their function. Mice mutant for *Pax3* and *Pax7* have been generated, but only *Pax3* exhibits any detectable segmentation phenotype. Further, neither line has been studied in detail for defects in epithelialisation, and the *Pax3/Pax7* mutant remains to be characterised in terms of segmentation.

In order to disrupt *Pax3* and *Pax7* in the chicken embryo, we explored the possibility of using RNA interference techniques to block transcription. Our first approach used the pRFPRNAi constructs developed by Das et al (Das *et al.*, 2006). The constructs coded for hairpin RNAs specific for the target gene. We found that low concentration electroporation sufficient for use in the neural tube was able to cause downregulation of *Pax3* expression (fig. A.6a). However, when using the higher concentrations of the pRFPRNAi constructs required for efficient transfection of the paraxial mesoderm, transfection caused severe damage to transfected cells, even in in control embryos (fig. A.6b). Confounding this effect, transfected cells did not cluster, making analysis of their phenotype difficult to assay (fig. A.6c, d).

As an alternative approach, we attempted to use antisense morpholinos. This approach has been used previously in young chick embryos to disrupt *Pax3* and *Pax7* (Basch and Bronner-Fraser, 2006), however we were unable to achieve detectable transfection with morpholinos, and no knockdown was detectable when a marker plasmid (pCIG) was included in the transfection (fig. A.6f, g).

In summary, for technical reasons we have been unable to analyse the loss of Pax3/7 function in our system. The effect of disrupting Pax3/7 remains an important question in understanding their role in somitogenesis.

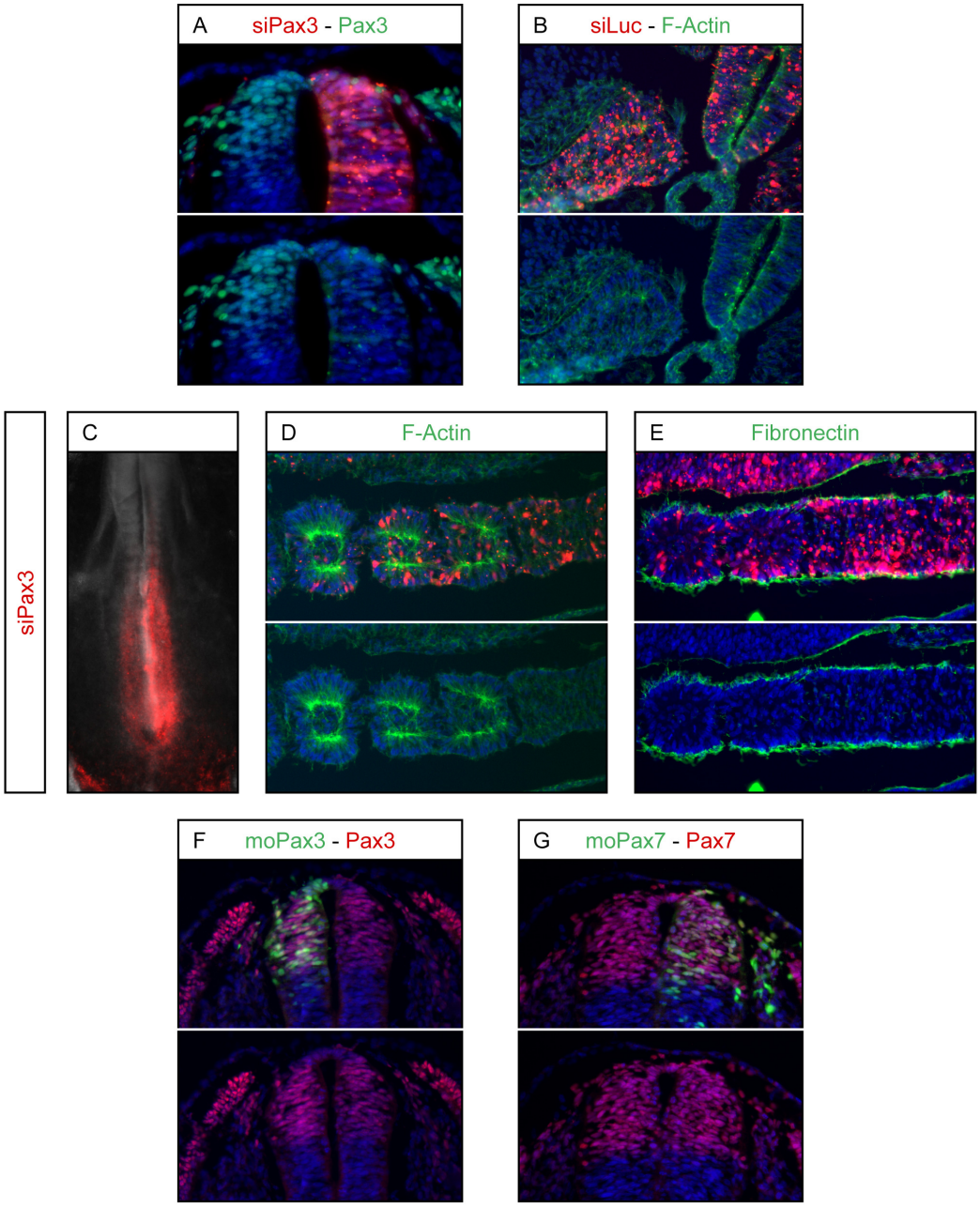


**Figure A.6: Strategies for Targetted Knockdown of *Pax3* and *Pax7***

A-E: Transfection with pRFPRNAi contstructs (Das *et al.*, 2006) containing hairpin sequences designed to target expression of Pax3 (siPax3) or luciferase (siLuc). Transfection in the neural tube induced effective downregulation of Pax3 protein (A, n=3). However, transfection of pRFPRNAi constructs into the PSM resulted in severe abnormalities in tissue and cellular morphology (B, n=4). Transfection of siPax3 constructs had minimal effects on F-actin or fibronectin. F-G: Transfection with morpholinos against *Pax3* and *Pax7* cotransfected with empty pCIG vector. No downregulation of Pax3 (F, n=4) or Pax7 (G, n=4) protein was detected.

# Appendix 7: Plasmids

## Expression Vectors

| Gene | Vector | Origin | Cloned By | Validation |
|---|---|---|---|---|
| GFP | pBI | In house | A. Ibrahim | Sequence/ Expression |
| Pax3 | pBI | In house | A. Ibrahim | Sequence/IHC |
| Pax7 | pBI | In house | A. Ibrahim | Sequence/IHC |
| Meso1[1] | pBI | Takahashi Lab | T. Watanabe | Sequence |
| Pax2[1] | pBI | Takahashi Lab | T. Watanabe | Sequence |
| Paraxis[2] | pBI | In house | D. Wright | Sequence |
| NCAM[3] | pBI | In house | D. Wright | Sequence/IHC |
| GFP | pCIG | McMahon Lab | S. Megason | Sequence/ Expression |
| Myf5 | pCIG | In house | A. Ibrahim | Sequence |
| rtTA (rtTA2s-M2)[1] | pCAGGS | Takahashi Lab | T. Watanabe | Sequence |
| siPax3 Hairpin | pRFPRNAi[4] | Wilson Lab | N. Van Hateren | Sequence |
| siPax7 Hairpin | pRFPRNAi[4] | Wilson Lab | N. Van Hateren | Sequence |

[1] Watanabe *et al.*, 2007

[2] Cloned from cDNA of *Paraxis* from S. Dietrich

[3] Cloned from HH12 chick cDNA library

[4] Das *et al.*, 2006

# RNA Probes

All plasmids validated by sequencing

| Gene | Vector | Origin | Cloned By |
|---|---|---|---|
| EphA4 | pBS-SK | Antin Lab | K. Patel |
| EphrinB2 | pBS-SK | Antin Lab | |
| Lef1 | | In house | A. Zagorska |
| Lfng | pBS-SK+ | Ish-Horowicz Lab | |
| Meso1 | pGEMT | Takahashi Lab | |
| Myf5 | pBS-KS+ | Dietrich Lab | |
| NCAM | pCRII-TOPO | In house | D. Wright |
| Paraxis | pBS-KS+ | Olsen Lab | |
| Pax1 | | Reshef Lab | |
| Pax2 | pBS-SK- | Nakamura Lab | |
| Pax3 | pBS | Dietrich Lab | |
| Pax7 | pBS | Dietrich Lab | |
| Snail2 | pBS-SK | Nieto Lab | |
| Tbx6 | pBS-SK- | In house | M. Schimpl |